MASTER OF SCIENCE

The Mechanism of Action of Lidocaine and Levobupivacaine on Human Cardiac Voltage-gated Na+ Channels NaV1.5

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The Mechanism of Action of Lidocaine and Levobupivacaine on Human Cardiac Voltage-gated Na^+ Channels $Na_V1.5$

By

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Abstract

Voltage-gated Na^+ channels (VGSCs) in nociceptive neurons are the molecular targets for local anaesthetics. However, due to the ubiquity of VGSCs in excitable tissues, they are prone to inhibition by local anaesthetics, including the cardiac $Na_V1.5$ subtype, which is also expressed in metastatic breast and colon cancer cells.The expression and function of $Na_V1.5$ facilitate invasion and migration of colon cancer cells. Local anaesthetics inhibit metastatic cancer cell invasion *in vitro* by inhibiting $Na_V1.5$ VGSCs, which may contribute to a direct mechanism by which regional anaesthesia during tumour resection reduces subsequent cancer recurrence. Metastatic colon cancer cells express adult and neonatal $Na_V1.5$ splice variants. Lidocaine and levobupivacaine are amide local anaesthetics that are widely used in the clinic. They have different physiochemical properties and thus differ in their pharmacokinetic and pharmacodynamic profile. We compared the mechanisms of action of these two local anaesthetics on adult and neonatal $Na_V1.5$ channelsrecombinantly expressed inhuman embryonic kidney cells usingwhole cell voltage-clamp.Lidocaine and levobupivacaine inhibited adult and neonatal $Na_V1.5$ in a state-dependent manner. Both drugs required the inactivated state of the channel for inhibition within the concentration range tested. Lidocaine and levobupivacaine stabilised the inactivated state, resulting in significant hyperpolarising shifts in the $V_{1/2}$ of inactivation, while neither affected the $V_{1/2}$ of activation. Levobupivacaine was approximately 10-fold more potent than lidocaine. The potency of both local anaesthetics was similar on adult and neonatal splice variants. The neonatal $Na_V1.5$ variant recovered more readily than the adult from lidocaine blockade. On the other hand, blockade by levobupivacaine of both splice variants, upon recovery from inactivation, was irreversible within the time scale examined. These findings are discussed in the context of their implications for cardiac toxicity and putative antimetastatic effects of local anaesthetics.

Dedication

To my loving parents, Rasem and Nadia, who have always offered their relentless support.

To my late grandfather, Khalifa and my grandmother, Fatima,who always encouraged me to seek knowledge.

To my sister, Nada, and my brothers Mohammed, Hamza and Omar who always stood by my side, even though we are miles apart.

I express my deepest gratitude to you all and thank you sincerely for all your efforts and support and for being the best family one can ever have.

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I would like to express the deepest appreciation to my supervisor Professor Tim Hales for all his guidance and support throughout my research.

I would also like to thank all the lab members for their help and for creating a lovely work atmosphere.

Declaration

I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The report is written in my own words and conforms to the University of Dundee's Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report.

Signature: 

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List of abbreviations

ATX: anemone toxin

EGTA: Ethylene glycol tetraacetic acid

FBS: Foetal bovine serum

GA: General anaesthetic

HEK: Human embryonic kidney cells

HEPES: 4-(2-hydroxyethyl)-peiperazineethanesulfonic acid

LA: Local anaesthetic

IC_{50}:The concentration which produces half maximal inhibition

IV: Intravenous

NCX: Sodium-calcium exchanger

NHE: Sodium hydrogen exchanger

PABA: para-aminobenzoic acid

TTX: Tetrodotoxin

VGSC: Voltage-gated sodium ion channel

$V_{1/2}$:The voltage at which activation/inactivation is half maximal

1. Introduction

1.1 The metastatic cascade

Cancer is a collective term for diseases that are characterised by uncontrolled cell growth, and the ability of these cells to migrate and invade other tissues. The initial defect in cancer is genetic damage to either tumour suppressor genes, or cancer promoting (oncogenes), which result in over-proliferation and primary tumerogenesis[1]. As the tumours increase in size its centre becomes hypoxic, which triggers the expression of angiogenesis (blood vessel formation) promoting genes[2]. The growing tumour is then able to receive nutrients and excrete metabolic waste via its own blood supply. Further growth favours the accumulation of more genetic changes leading to more aggressive behaviours whereby the cancer cells start invading surrounding tissues[2]. Invasion is initiated by detachment from the primary tumour, followed by increased motility and digestion of surrounding basement membrane through the action of proteolytic enzymes[2,3] _ENREF_2. Once the cells reach the circulation in a process called intravasation, they can spread around the body and migrate via the blood or lymph. Migrating cells are subject to metabolic and physical stress and most cells undergo anoikis, a specific from of apoptosis[2]. However, surviving cells can lodge into distant sites in a process called extravasation, where they either lie dormant for years or form secondary tumours immediately. Metastasis is the overall process of secondary tumour formation and it is the main cause of death in cancer patients.

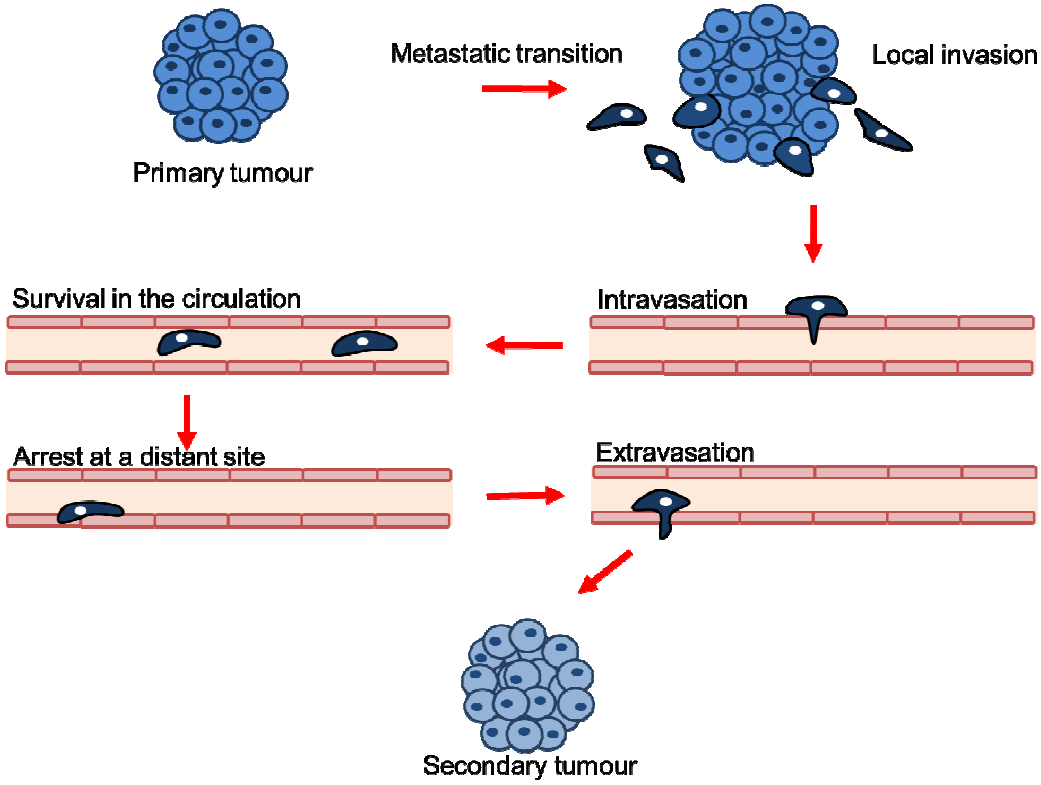

Figure 1.1 Diagrammatic illustration of the metastatic cascade highlighting the main stages from primary tumorigenesis to formation of secondary tumours.

1.2 The benefit of local anaesthesia in tumour resection

For many cancers, the first-line treatment is surgery. However, surgery can liberate metastatic cancer cells into the circulation, a phenomenon termed the showering effect[4,5] _ENREF_4. Those cells can then either re-seed or self-seed and form secondary tumours[5-7], thus increasing the incidence of recurrence. According to several retrospective studies, the use of local anaesthesia during tumour resection has the benefit of improving surgery outcome and lowering the incidence of recurrence[8-11]. Accordingly, there are ongoing clinical trials investigating the effect of local anaesthesia on cancer recurrence in operable tumours.To date, there is only one phase 3 randomised controlled clinical trial to assess the effects of blockade of VGSCs by lidocaine in India. The outcomes under investigation are disease free interval and long-term survival in operable breast cancer, which will be assessed in 2021(ClinicalTrials.gov Identifier: NCT01916317). It is also

noteworthy that a number of retrospective studies to assess anaesthetic technique showed no clear correlation between local anaesthesia and cancer survival or recurrence[12-14] _ENREF_12. Such results, however, must be carefully interpreted, as other confounding variables were not ruled out and the causes of death were not indicated. The benefit of local anaesthesia to cancer survival is said to be a result of the following mechanisms.

Lowering the general anaesthetic and opioid dose: General anaesthetics (GAs) have an immunosuppressive effect that results in compromising the patient's cell-mediated immune response and increases the likelihood of positive circulating metastatic cells[15]. Most GAs cause cytotoxicity to natural killer cells[16]. Similarly, opioids, particularly morphine have an inhibitory effect on humoral and cellular immune functions in humans[15]. Considering the potential deleterious effects of both GAs and opioids on cancer surgery outcome, minimising their required dose might improve outcome and recovery. This can be achieved via administration of local anaesthetics.

Effects on tumour progression: Amide-linked local anaesthetics, such as lidocaine and ropivacaine have anti-inflammatory actions via inhibition of c-src phosphorylation, which is pivotal for signalling the epithelial-mesenchymal transition[17]. The latter is necessary for solid tumour metastasis. Inhibition of c-src phosphorylation also inhibits intracellular adhesion molecule 1 (ICAM1), which facilitates extravasation[18]. Persistent inflammation is said to exacerbate carcinogenesis and mediates the cellular processes that are necessary for the development of the aggressive neoplastic phenotype. As such, the anti-inflammatory actions of LAs may reduce the risks of cancer surgery. Figure 1.2 provides a summary of the mechanisms by which local anaesthesia may affect surgery outcome and cancer recurrence.

Nevertheless, the aforementioned mechanisms are indirect. There is mounting evidence to suggest that the benefits of LAs to reduce tumour metastasis after surgery is via inhibition of voltage-gated sodium ion channels (VGSCs), which are upregulated in many metastatic cancer cells.

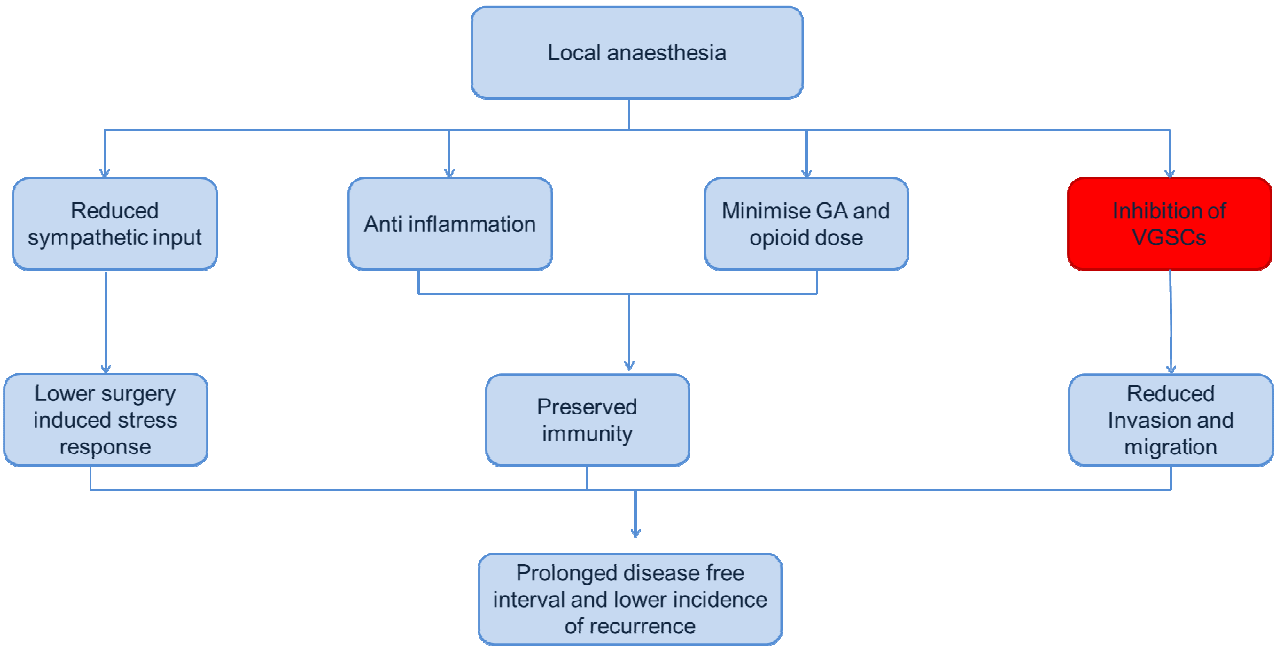

Figure 1.2 Summary of the benefits of local anaesthesia in tumour surgery indicating direct and indirect proposed mechanisms.

1.3 The role of VGSCs in metastasis

1.3.1 VGSCs: structure and function

VGSCs are multimolecularprotein complexes that span the plasma membrane. They consist of an obligate pore-forming alpha-subunit that is 260 KDa and is encoded by 9 different genes (SCN1A-SCN11A), in addition to one or two auxiliary beta-subunits of 30-40 KDa that are encoded by the genes SCN1B-SCN4B[19].

The alpha subunit comprises four homologous domains (D1-D4), each consisting of 6 transmembrane segments (S1-S6). The fourth alpha-helical segment (S4) is a voltage-sensor, which contains positively-charged amino acid residues, mainly arginines, at every third position[20]. The S5, S6 and the connecting pore-loops link together to from the pore domain. The pore domain contains the selectivity filter, consisting of four residues (DEKA)[21],

which is the narrowest region of the pore that selectively allows the passage of hydrated sodium ions. New structural data revealed the existence of additional lateral lipid-filled opening, termed, fenestrations, that link the membrane to the channel pore. These fenestrations are important access pathways for small hydrophobic drug molecules[21].

The inactivation gate consists of the intracellular loop between D3 and D4[22,23] _ENREF_19. Three amino acids, namely isoleucine, phenylalanine and methionine (IFM) form a key motif for fast inactivation. Channels with mutated IFM motifs show slowed inactivation, which can be restored using peptides containing this motif. The inactivation gate acts as a hinged lid and folds inwards to occlude the pore upon fast inactivation[24]. The activation gate is made up of four residues from each of the intracellular ends of the S6 segment. This structure provides an intracellular cavity and it is highly conserved in VGSCs[21].

VGSCs display voltage-dependent gating. At rest (under hyperpolarised potentials), the probability of the channel to exist in an open state is low, and thus it predominantly assumes a closed conformation, where sodium ions are denied entry. Activation occurs in response to depolarisation, which is mediated via the outward movement of the voltage sensor that subsequently opens the pore and allows sodium ions into the cytosol[25]. Within 1-2 ms, the channel assumes a non-conducting state and the inactivation gate occludes the pore preventing further sodium ions influx. The rate of inactivation varies according to the subtype of expressed alpha subunit. It is suggested that fast inactivation occurs as a consequence of activation and hence the two processes are coupled. There also exists a second inactivated state (slow-inactivation), which is initiated by prolonged depolarisations[26]. There also exists a persistent component of the Na^+ current (I_{NaP}), which unlike the transient current, is resistant to inactivation for seconds. The I_{NaP}may result from conformational changes to the channel as a result of post-translational modification, e.g. phosphorylation. More importantly, it has been shown to be upregulated in conditions such as hypoxia, which predominates in cancer cells and therefore may contribute to the overall increase of intracellular Na^+[27].

In addition to voltage across the membrane, the conformation of VGSCs is influenced by the presence of beta-subunits, which modulate gating, post-translational modifications, such as phosphorylation; and the presence of pharmacological agents[21]. Some VGSCs also display mechanosensitivity, which influences their kinetics and voltage-dependence of activation and inactivation[28].

The expression of VGSCs is tissue-specific. $Na_V1.1$, $Na_V1.2$ and $Na_V1.3$ are mainly expressed in the central nervous system, while $Na_V1.6$ is expressed in both, central and peripheral nervous systems[21]. $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are primarily restricted to the peripheral nervous system[21]. $Na_V1.4$ and $Na_V1.5$ are expressed in skeletal and cardiac muscles, respectively[21]. The canonical role of VGSCs is action potential initiation and propagation along nerve and muscle fibres, which underlie multiple functions, from nociception to muscle contraction.

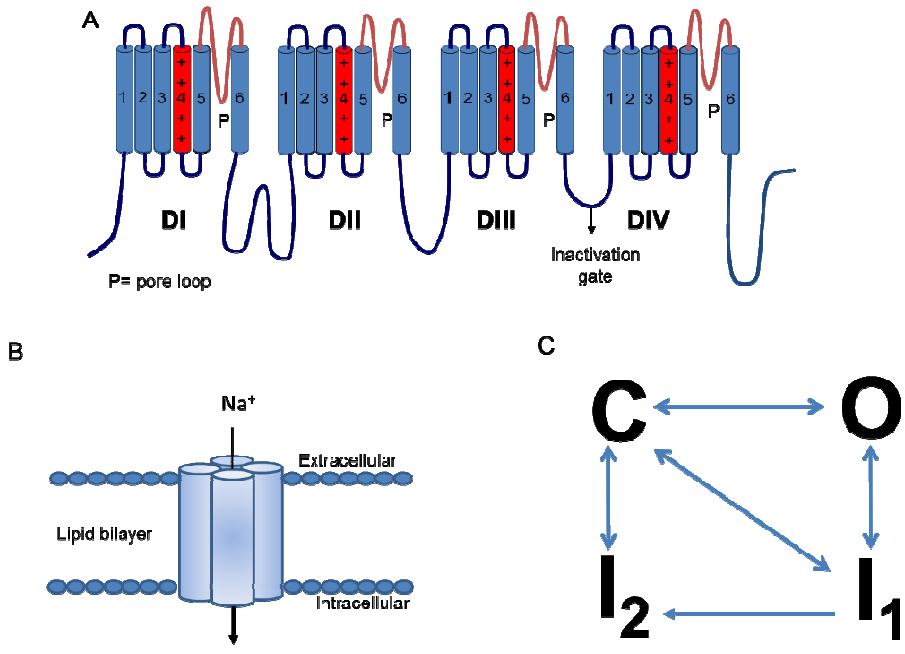

Figure 1.3 Structure and gating of voltage-gated sodium channels (VGSCs). (A) Topology of the alpha subunit of VGSCs, showing the four domains (I-IV), with 6 transmembrane segments. The S4 indicated in red is the voltage sensor. The pore is made up from segments 5 and 6. (B) Drawing of the alpha subunit structure showing the three dimensional arrangement of the domains (I-IV) within the lipid bilayer. (C) Gating model of VGSCs illustrating the different conformational states. C= closed, O= open, I1= fast inactivation and I2= slow inactivation.

The SCN5A encoding the α-subunit of the cardiac VGSC undergoes alternative splicing, resulting in isoforms with unique functional properties. In metastatic cancer cells, two splice variants are identified, namely adult and neonatal[29] that differ in exon 6 that correspond to the S3, S3-S4 linker and S4 that forms the first voltage sensor, resulting in a substitution of 7 amino acids, as illustrated in Figure 1.4. One charge substitution occurs at position 211, where an aspartate is substituted for a lysine in the neonatal splice variant, which was shown to be the main determinant of the functional differences between the adult and neonatal splice variant. The neonatal $Na_V1.5$ displays slower inactivation and recovery from inactivation kinetics, as well as a more depolarised $V_{1/2}$ of activation, compared to the adult[30]. Despite the conservation of the LAs binding site, the differences in the kinetics may indirectly influence the pharmacology of these splice variants,given that LAs are state-dependent blockers.

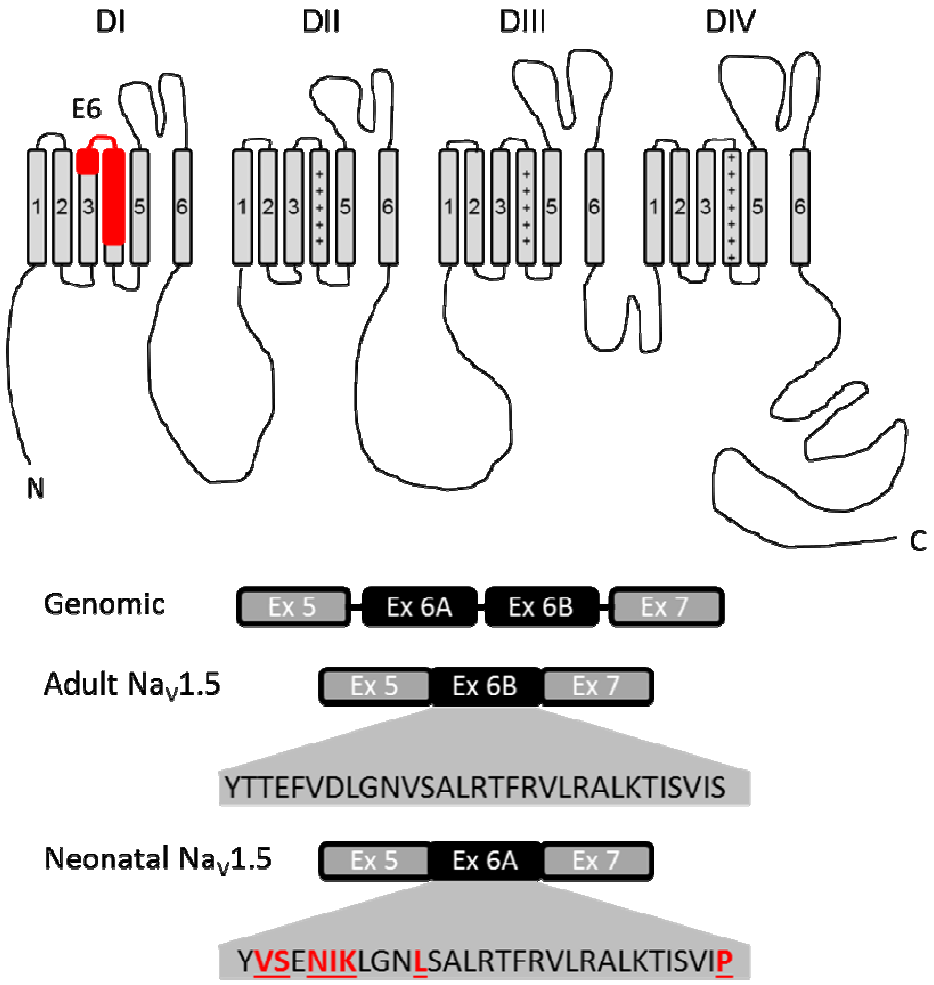

Figure 1.4 Schematic representation of the Na$_V$1.5 α-subunit highlighting the difference in amino acids between the adult and neonatal splice variants. The top panel illustrates the region in the translated protein that corresponds to the site of splicing. The bottom panel shows exon 6, which is affected by the splicing. Usage of either exon 6B or exon 6A results in mRNA encoding the adult Na$_V$1.5, or the neonatal Na$_V$1.5, respectively. The non-conserved amino acids in the neonatal splice variant are highlighted in red.[29]

1.3.2 Noncanonical roles of VGSCs

VGSCs are identified in many non-excitable cells where they participate in a plethora of cellular processes from proliferation to migration[31]. Such processes are vital for embryogenesis during development, as well as tissue repair. Various studies indicate the necessity for VGSCs in heart and brain development in mice[32-34]. In addition, mutations in voltage-gated ion

channels are associated with increased risk of craniofacial and limb defects[35-37]. Foetal exposure to VGSCs inhibitors also increases the risk of birth abnormalities[38-40]. The non-excitable roles of VGSCs would explain the incentive for their upregulation in cancer cells to acquire a highly invasive phenotype.

1.3.3 VGSCs as key regulators of cancer metastasis

Various subtypes VGSC-α subunit are functionally expressed in several cancer cell lines, particularly those that are metastatic (Table 1).

Cancer type	**VGSC-α subtypes**
Colon	$Na_V1.5$ (adult and neonatal)[41]
Breast	$Na_V1.5$ (neonatal) and $Na_V1.7$[42,43]
Prostate	$Na_V1.9$ and $Na_V1.7$[44,45]
Cervix	$Na_V1.6$[46]
Ovary	$Na_V1.5$ and $Na_V1.1$[47]
Small-cell and non-small-cell lung cancer	$Na_V1.7$ and $Na_V1.9$[48,49]
Lymphoma	$Na_V1.9$[50]
Neuroblastoma	$Na_V1.5$[51]
Melanoma	$Na_V1.6$[52]
Mesothelioma	$Na_V1.2$, $Na_V1.6$ and $Na_V1.7$[53]

Table 1.1 Subtypes of VGSCs found in cancer cells.

Numerous studies indicate that VGSC function contributes to several metastatic cell behaviours. Gene silencing studies showed that downregulation of VGSC through small interfering RNAs reduced invasion and migration in several cancer cell lines[41,43,44,54]. Interestingly, gene silencing had no effect on cell proliferation in all of these studies. Conversely, overexpression of VGSCs favours metastatic cell behaviour by increasing invasion. A study was carried out on weakly metastatic prostate cancer cells, where the $Na_V1.4$ was overexpressed and it resulted in an increase of invasiveness[55].

Pharmacological inhibition of VGSCs produces the same result. The highly selective neurotoxin TTX inhibited various metastatic cell behaviours in multiple human and rodent cancer cell lines[41,42,47,48,55-59]. Moreover, local anaesthetics, such as lidocaine and ropivacaine have been shown to be potent inhibitors of invasion of metastatic colon cancer cells[29,41]. On the other hand, VGSC activators, such as veratridine and anemone toxin (ATX) have been shown to enhance invasiveness and motility[41,60]. VGSC blockers have also been proven effective in inhibiting metastasis in animal models. When directly applied to the primary tumour, TTX was shown to significantly reduce lung metastasis in the Dunning rat model of prostate cancer[61]. No effects were noticed on cell proliferation and the animals lived significantly longer. Additionally, systemic administration of RS100642[62], an analogue of mexelitine and an inhibitor of TTX-R VGSCs resulted in prolonged survival[63]. Downregulation of $Na_V1.5$ in an orthotopic breast cancer model reduces metastasis to liver, lungs and spleen[64].

The notion that VGSC activity is necessary for metastasis is backed up by evidence from clinical studies. It was shown that intra-tumour sodium levels are elevated in metastatic cells compared to none metastatic ones[65-67]. Furthermore, high levels of expression of Na_v mRNA are associated with increased risk of metastasis and higher incidence of recurrence[42,68].

1.3.4 Pharmacology of VGSCs

VGSCs are modulated by a variety of neurotoxins, including the pore blocker tetrodotoxin (TTX), saxitoxin, μ-conotoxin, betrachotoxin and peptides

derived from scorpion and tarantula venom[21]. They are classified according to their TTX-sensitivity. TTX-sensitive subtypes are blocked by nanomolar concentrations of TTX, and these include $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_v1.4$, $Na_v1.6$ and $Na_V1.7$. Whereas, TTX-resistant subtypes are only blocked by micromolar concentrations of TTX, including $Na_v1.5$, $Na_v1.8$ and $Na_V1.9$[21]. A wide range of clinically used drugs also modulate VGSC function to treat several pathologies including pain, arrhythmia and epilepsy. Local anaesthetics (LAs) are one example; they are used to block nociceptive nerve fibres to induce anaesthesia and analgesia. They are also used to interfere with cardiac VGSCs to treat arrhythmias. Most VGSC inhibitors are state-dependent blockers, i.e. they display differential affinities for different channel conformations[69]. Such properties enhance their selectivity for inactivated channels that are more prevalent in pathological conditions and thus minimising their side effects.

1.4 Local anaesthetics

LAs are a class of drugs that are widely used in the clinic as analgesics and antiarrhythmics. They are broadly classified into ester-linked and amide-linked LAs according to their chemical structure; and the latter are usually preferred, as ester compounds are metabolised to para-aminobenzoic acid (PABA), which causes anaphylactic responses in some individuals. Amide LAs also have a longer half-life, as they escape metabolism by plasma pseudocholinesterases. Lidocaine, levobupivacaine and ropivacaine belong to the amide-linked group. These compounds share a chemical structure of a phenyl ring, an amide-linkage and a peripheral tertiary amine. The pharmacokinetic and pharmacodynamics profile of LAs is largely determined by three properties: (1) $pK_{a,}$ (2) hydrophobicity and (3) protein binding. LAs are amphipathic, i.e. they exist in neutral and chargedforms and the proportion of molecules in the neutral membrane-permeant form at physiological pH is dependent on their pKa. This property dictates the rate of onset of analgesia and thus LAs with a relatively lower pK_a will have a faster onset. Hydrophobicity determines lipid partitioning, which dictates potency. At the nerve level, this influences the diffusion rate across the myelin sheath and is affected by nerve diameter. At the molecular level, however, this

property influences the access pathway to the channel and may influence the ability of the LA molecule to unbind or remain trapped within hydrophobic pockets. Lastly, protein binding dictates the duration of action of LAs, as free circulating drug is more slowly made available for metabolism. These properties are crucial when selecting LAs for specific therapeutic purposes, as well as rational drug design of LA derivatives. The previously discussed properties are summarised in the table below for three of the most widely used LAs in the clinic.

Local anaesthetic	**pKa**	**% Ionised (at pH 7.4)**	**Partition coefficient (lipid solubility)**	**% Protein binding**
Lidocaine	7.9	76	366	64
Ropivacaine	8.1	83	775	94
Levobupivacaine	8.1	83	3420	97

Table 1.2 Physicoochemical properties of clinically used local anaesthetics.[70]

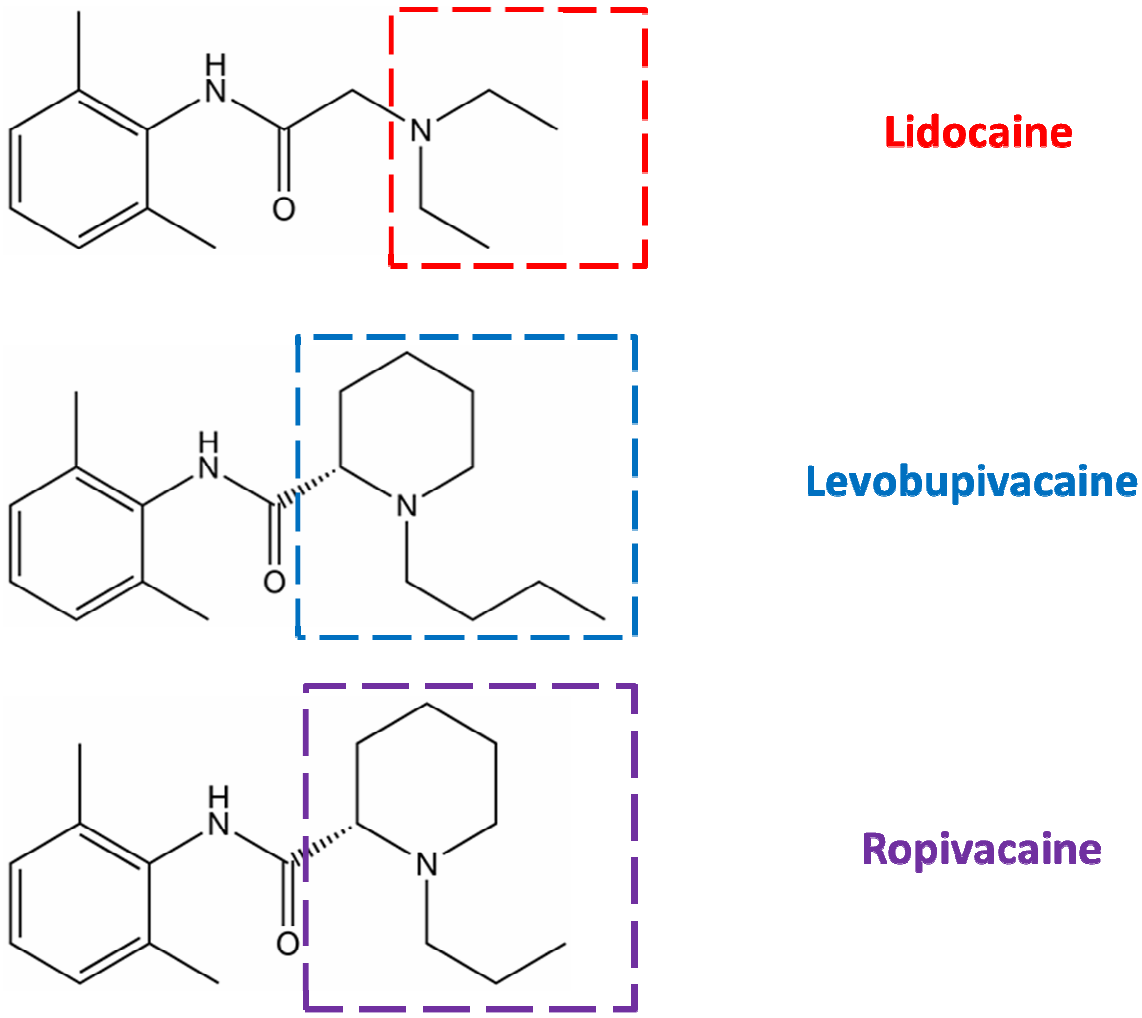

Figure 1.5 The chemical structure of lidocaine, levobupivacaine and ropivacaine highlighting the tertiary amine group, which is unique in each of the three molecules.

As stated previously, the pharmacological effects of LAs come about through state-dependent inhibition of VGSCs[71]. They alter gating by stabilising the inactivated conformation. This mechanism was proposed by Hille in the modulated receptor hypothesis, which entails that the LA binding site is modulated upon depolarisation; and the open and inactivated conformations have higher affinities for LAs than the resting state[72]. This particular mode of action is what makes LAs suitable for inhibiting repetitive neuronal firing in pathological conditions. Two aromatic residues (phenylalanine 31 and

tyrosine 20) in the middle of the D4-S6 segment were first identified as crucial residues for LA binding[73]. Those studies were carried out on rat brain $Na_V1.2$ α-subunit using alanine scanning. Further site mapping studies identified other regions on D1, D2 and D3 that influence LA binding[74]. Amino acid residues in those regions are highly conserved in all 9 isoforms of the α-subunit. LAs can access their binding site either via fenestrations within the lipid bilayer, or intracellularly after traversing the lipid bilayer. Interestingly, the cardiac VGSCs ($Na_V1.5$) contain a unique threonine residue in the D4-S6 segment, which provides an additional hydrophilic pathway through the external surface. Quaternary lidocaine (QX-314), which is membrane-impermeant, is able to bind cardiac VGSCs from the either side of the membrane, however, it can only bind neuronal and skeletal VGSCs when applied from the inside[75]. This raises possibilities for selectively targeting the cardiac VGSCs with QX-314. Lidocaine, ropivacaine and bupivacaine inhibit cardiac and neuronal VGSCS in a state- and use-dependent manner[73,76-78]. The physiochemical properties of these drugs influence the concentration-dependence, use-dependence and recovery from block from VGSCs, indicating that modest difference may reflect on their differential interactions with neuronal and cardiac VGSCs. Moreover,differential gating of $Na_V1.5$ and $Na_V1.7$ determine the mode of action of lidocaine and other antiarrhythmic, such as mexiletine[78].

LA toxicity can occur when sufficient amounts are absorbed into the circulation and they affect excitable tissues such as the CNS and the heart. It has an incidence of 1 in 1000 in peripheral nerve block[79]. The maximum tolerated dose for lidocaine and levobupivacaine are 3 mg/kg and 2.5 mg/kg, respectively. To minimise systemic toxicity, LAs are usually co-administered with a vasoconstrictor, such as adrenaline. Detailed understanding of the mechanism of action of LAs pre-clinically is of paramount importance, in order to carefully select candidates that are tailored to ameliorate the post-operative recurrence of cancer following surgical excision.

1.5 VGSCs blockers as anti-metastatic drugs

The previous evidence inevitably directs our attention to Na^+ channel blockers as potential metastatic tumour suppressors. Re-purposing of VGSCs inhibiting drugs may provide an opportunity to rapidly impact clinical practice. Yang *et al* demonstrated this approach using phenytoin, an anti-convulsant, to reduce invasion of human breast cancer in an animal model[68]. In order to maximise the therapeutic benefit of re-purposing, drug candidates have to be thoroughly selected. Ideal candidates would selectively inhibit VGSCs on cancer cells with minimal off-target effects. Given the involvement of VGSCs in many channnelopathies, the search for selective ligands is a popular area of research, particularly for analgesia and pain management. However, drug binding sites on VGSCs are highly conserved[21], in addition to their ubiquitous expression; and thus selective targeting has proven to be a major challenge. Nonetheless, the expression of neonatal isoforms in colorectal and breast cancer cells can be exploited to achieve selectivity, given that this isoform is absent in cardiac tissue of adults. The neonatal $Na_V1.5$ differs from the adult in 7 amino acids in exon 6 as a result of alternative splicing[29,30]. This region corresponds to the voltage-sensor in domain I on the translated protein. The neonatal isoform thus differs from the adult in the voltage-dependence of activation, as well as the kinetics of inactivation. However, no differences in drug binding sites are present. Accordingly, LAs such as ropivacaine, which is widely used in the clinic, displays no selectivity for either isoform[29]. Antibody therapy, may offer an opportunity to selectively target the neonatal isoform. There has been success in producing neonatal-$Na_v1.5$-specific polyclonal antibodies[80].

Furthermore, state-dependence, an intrinsic property of most clinically used VGSC blockers can be exploited to achieve selectivity. As mentioned previously, VGSCs blockers, such as LAs have a preference for the inactivated state, i.e. they bind with a higher affinity to activated and inactivated channels as opposed to resting ones. Under physiological conditions, VGSCs cycle through the different states and the inactivated state is rarely assumed, i.e. it has a very short lifetime. However, under pathological conditions, including pain, arrhythmia and epilepsy, action

potential frequency increases and thus inactivation occurs more frequently and has a longer lifetime. Consequently, inactivation state-dependent VGSC blockers will readily bind to the channel and inhibit its function. In cancer cells, the membrane potential is relatively more depolarised. For instance, metastatic colorectal cancer cells (SW620) have a membrane potential of -40 mV[29]. At such potentials, the majority of the channels are in the inactivated state and only a small proportion transition to the activated state according to the theoretical window current, which corresponds to the range of potentials at which tonic current is present. This is evidenced by the inhibition of invasion *in vitro* and *in vivo*by TTX, which blocks the pore of the channel and denies Na^+ influx[41,61]. In addition, pressure can contribute to the activation of the channels as the $Na_V1.5$ are mechanosensitive[28]. Given the large proportion of inactivated channels at -40 mV, theywill be more susceptible to blockade by drugs, such as LAs. In addition to that, cancer cells are unable to fire action potentials and as a result are unable to recover from inactivation at rest. Such conditions would favour long-term inhibition by drugs where the function of VGSCs can only be reinstated once new channels are inserted into the membrane.

It is known that VGSCs in prostate (Mat-LyLu) cancer cells are auto-regulated in a positive feedback loop, whereby the activity of the channel promotes further Na^+ influx and insertion of new channels into the membrane[58]. Therefore, chronic inhibition by local anaesthetics (or other VGSC blockers) would serve the benefit of supressing both channel activity and expression. This anti-metastatic therapeutic strategy would have a twofold advantage.

Lidocaine, in particular, is a promising candidate, as it can be given intravenously either peri-or post operatively. Pre-clinical studies are required to determine the safest local anaesthetic with regards to systemic toxicity. Low doses of lidocaine are likely to be effective based on electrophysiological data showing that cancer cells have a relatively more depolarised resting membrane potential, at which most VGSCs will be in the inactivated state. Inability to recover from inactivation may render the blockade irreversible and thus long-term inhibition is possible with this

strategy. Cancer patients would benefit from local anaesthetic derivatives that are tailored to target inactivated VGSCs in metastatic tumour cells that can be used at low doses to minimise toxicity. Additionally, isoform-selective inhibitors would offer novel therapy for metastatic cancer.

1.6 Project aims

This study aims at providing a comparative analysis of the effects of lidocaine and levobupivacaine on two splice variants of $Na_V1.5$ found in metastatic colon cancer cells, namely adult and neonatal $Na_V1.5$. Human embryonic kidney (HEK293) cells are used as heterologous expression systems to characterise the effects of lidocaine and levobupivacaine under whole-cell voltage clamp. The preclinical investigation will highlight key pharmacodynamic aspects of lidocaine and levobupivacaine that can be translated for optimal selection of local anaesthetics as anti-metastatic drugs.

2. Materials and methods

2.1 Plasmid preparation

Adult and neonatal $Na_V1.5$ were subcloned into pcDNA3.1 vector (Invitrogen). The SCN5A, originally known as human heart 1 (HH1) cDNA was a gift from Alfred George. To create the neonatal $Na_V1.5$ pcDNA3.1 vector, mRNAfragments containing exon 6A from SW620 cells were replaced into the same region of the pcDNA3.1-$Na_V1.5$[29].

2.2 Cell culture and transfection

Human embryonic kidney (HEK293) cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Paisley, UK). Confluent cells were sub-cultured every 3-4 days and plated onto 35 mm dishes at low density for electrophysiological experiments. All cells were

maintained in an incubator at 37^{o}C and 5 % CO_2, except those to be transfected using the calcium phosphate precipitation method, which were kept at 3.5% CO_2.

HEK293 cells were transiently transfected using the calcium phosphate precipitation method with human SCN5A (adult or neonatal) in pcDNA3.1 vector at 1 µg/dish, along with cDNA for green fluorescent protein at 0.1 µg/dish[29]. GFP was used to identify transfected cells.The proportion of transfected cells was approximately 60% per dish, 90 % of which on average had voltage-activated Na^+ currents. Cells were washed from the transfection media on day 1 post-transfection and were recorded from on days 2 and 3. A minimum of 3 transfected dishes contribute to n numbers, which correspond to the number of cells from which data was obtained.

2.3Electrophysiology

Voltage-activated Na^+ currents were recorded from HEK-293 cells transiently expressing adult or neonatal $Na_v1.5$ using the whole-cell voltage-clamp technique. Currents were recorded using an Axopatch 200B patch-clamp amplifier, low-pass filtered at 2 KHz, digitised by Digidata 1320A interface, sampled at 4 KHz and acquired using pClamp8 software (Molecular Devices, CA, USA). Pipettes were formed from borosilicate glass capillaries and had resistances between 1.5 and 3 MΩ when filled with intracellular solution, containing (in mM) the following: 130 CsCl, 15 NaCl, 2 $MgCl_2$, 10 EGTA and 10 HEPES. CsCl was used to block K^+ currents. The extracellular solution contained (in mM) the following: 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose and 10 HEPES). Recordings were only accepted from cells with the following parameters: peak current amplitudes < 5 nA,series resistance < 5 mΩ and compensatedby ≥ 85 %, and capacitive artefacts with amplitudes < 30% of the peak current. This selection criteria ensures that voltage errors are less than 4 mV. All solutions were bath applied and recordings were taken under continuous perfusion of bath solutions at room temperature. No corrections were made for the junction potential.

A holding potential of –80 mV was used in all protocols unless stated otherwise.

To examine the voltage-dependence of activation, a series of 25-ms voltage steps were applied between voltages of -80 mV to +70 mV, in 10 mV increments, with an interpulse duration of 5 seconds. The voltage-dependence of steady-state inactivation was examined using 100 ms prepulses to voltages ranging from -140 mV to -10 mV, in 10 mV increments, followed by an activation test-pulse to 0 mV. In order to examine use-independent (resting state) and use-dependent block by local anaesthetics, the following protocol was used. Pulses to 0 mV at a frequency of 1 Hz were applied under three different holding potentials (-80, -90 and -120 mV). Currents were recorded initially under control conditions. The local anaesthetics were then applied for 2 minutes to reach steady-state block, after which another set of recordings were taken. To examine recovery from inactivation and block by local anaesthetics, a step to -120 mV was applied at time intervals between 10 to 300 ms,in 10 ms increments. This step was used to remove inactivation and hence cause drug unbinding. Currents were then activated by a pulse to 0 mV before returning to the holding potential.

2.4Statistics and data analysis

Current amplitudes were measured using pClamp8 (Molecular devices, CA, USA). Plots of the voltage-dependence of activation were derived from current-voltage relationships. The driving force at each holding potential (up to +20 mV) was determined from the theoretical Na^+ equilibrium potential, in order to calculate Na^+ conductance. Conductances were normalised to the respective peak values in each cell. Similarly, for the voltage-dependence of inactivation currents were normalised to peak amplitude values for each cell. Activation and inactivation data were fitted with Boltzmann functions.

$$Y = Minimum + \frac{(Maximum - Minimum)}{1 + Exp\left(\frac{V50 - x}{Slope}\right)}$$

Where Y is conductance for voltage-dependence of activation, or current for voltage-dependence of inactivation , X is voltage, minimum is a constant made equal to zero, maximum is a constant made equal to 1, $V_{50}$is the voltage at which activation is half maximal and slope is the steepness of the curve.

Concentration-response data were fitted with a logistic function. Average parameters were determined from fits to data acquired from individual cells. For recovery from inactivation and block by local anaesthetics, currents were normalised to peak values and fitted against the recovery time by single or double decay functions. Time constants were determined from data obtained from individual cells.

Weighted time constants were calculated as follows:

$\tau_w = (\tau_f \times Amp_f) + (\tau_s \times Amp_s)$

Where τ_w is the weighted tau, τ_s and τ_s are the fast and slow time constants, respectively; and Amp_f and Amp_s are the amplitudes of the fast and slow components, respectively. All data are presented as mean ± SEM. Statistical comparisons were performed using the paired or unpaired t-test, or a one-way ANOVA, as appropriate. Differences were considered significant at $p \leq 0.05$. Fitting and statistical analyses were performed using GraphPad Prism software (La Jolla, CA, USA).

3.Results

3.1 Inhibition of adult and neonatal NaV1.5 by lidocaine and levobupivacaine

We compared the effects of lidocaine and levobupivacaine (0.3 -30 μM) on currents mediated by recombinant adult and neonatal $Na_v1.5$ expressed in HEK293 cells. Currents were elicited by a 0 mV step from a holding potential of -80 mV before and after drug application to the recording chamber. The resulting cumulative concentration-response relationships are shown in Figure 3.1. Both lidocaine and levobupivacaine caused potent inhibition of $Na_V1.5$-mediated currents. However, there were no differences between splice variants with respect to the concentration dependence of inhibition by either local anaesthetic (Table 3). Levobupivacaine was approximately 10-fold more potent than lidocaine, with IC_{50} values of 1.0 ± 0.2 (n=4) and 3.0 ± 0.6 μM (n=3) and 20 ± 9.0 and 17 ± 5.0 μM, for levobupivacaine and lidocaine on adult and neonatal $Na_V1.5$, respectively.

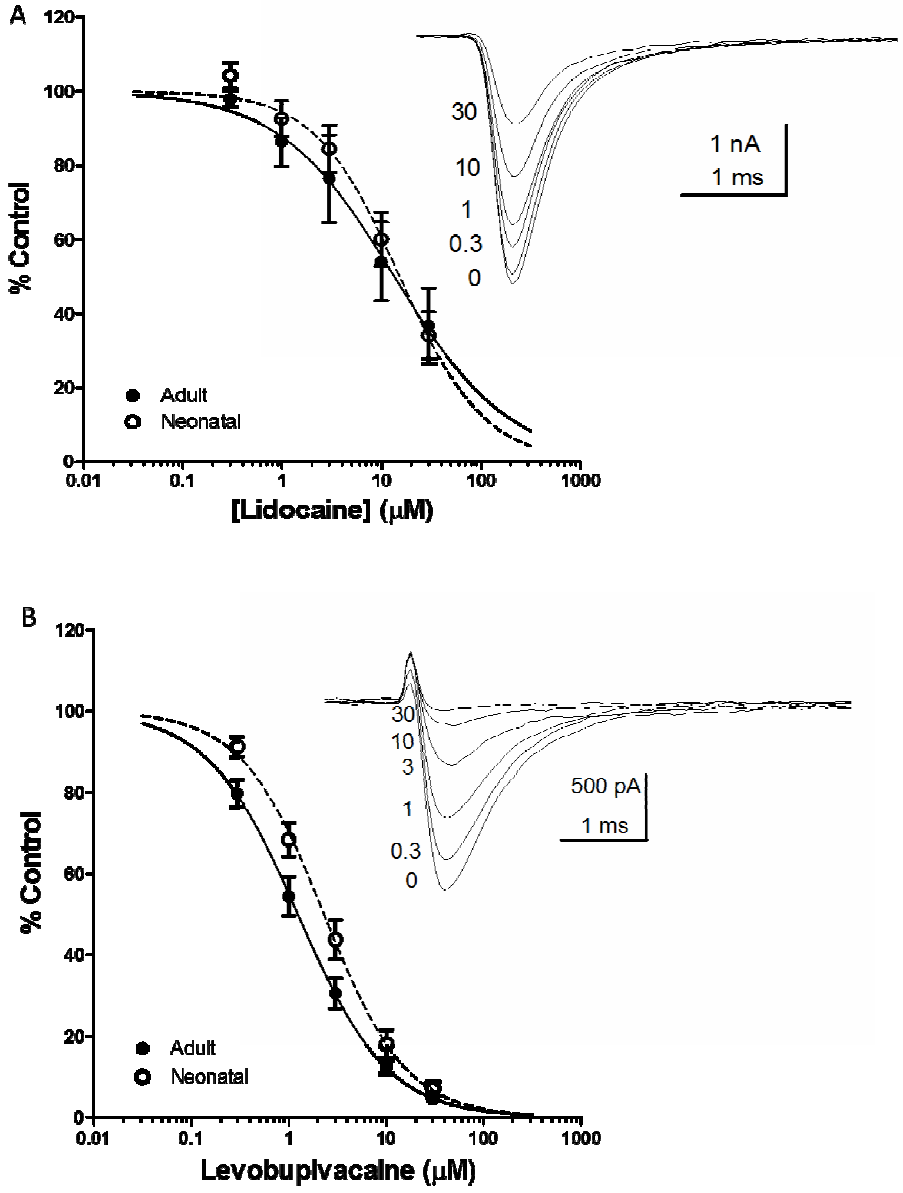

Figure 3.1 Concentration-response relationship for lidocaine (A) and levobupivacaine (B) on adult and neonatal $Na_V1.5$. Recordings were made from HEK293 recombinantly expressing adult or neonatal $Na_v1.5$. Currents were elicited by an activating step to 0 mV for 25 ms at 1 Hz, from a holding potential of -80 mV. The concentration response relationships were fitted using a logistic function. Average parameters were obtained from data recorded from individual cells (Table 3.1). Insets represent exemplary currents mediated by adult $Na_V1.5$ in the presence of lidocaine or levobupivacaine (0.3-30 μM).

Na_V Isoform	**IC_{50} (μM)**		**n**
	Lidocaine	Levobupivacaine	
Adult	20 ± 9.0	1 ± 0.2	4
Neonatal	17 ± 5.0	3 ± 0.6	4

Table 3.1 Summary of IC_{50} values of lidocaine and levobupivacaine on adult and neonatal $Na_V1.5$. IC_{50} values were taken from logistic fits to data from individual cells. Statistical analyses were performed using an unpaired t-test. No significant differences were observed between the potencies of both LAs on either splice variant.

3.2 The effects of lidocaine and levobupivacaine on the voltage-dependence of activation of adult and neonatal $Na_V1.5$

We tested the effects of lidocaine and levobupivacaine on both splice variants to determine whether they differentially influence the voltage-dependence of activation. Paired recordings were taken before and after drug application. We used a series of voltage steps from -80 mV to +70 mV and recorded the corresponding currents to determine the current-voltage relationship. In agreement with previous reports[29,30], the neonatal splice variant of $Na_V1.5$ had a more depolarised voltage-dependence of activation (-26 ± 1 mV, n=11), compared to the adult variant (-33 ± 2 mV, n=14). The derived activation curves are shown in Figure 3.2.B and a summary of the electrophysiological parameters is provided in table 3.2. Both lidocaine and levobupivacaine suppressed currents mediated by adult and neonatal $Na_V1.5$ over a range of potentials from -80 mV to +20 mV. However, neither drug affected the $V_{1/2}$ of activation (Table 3.2).

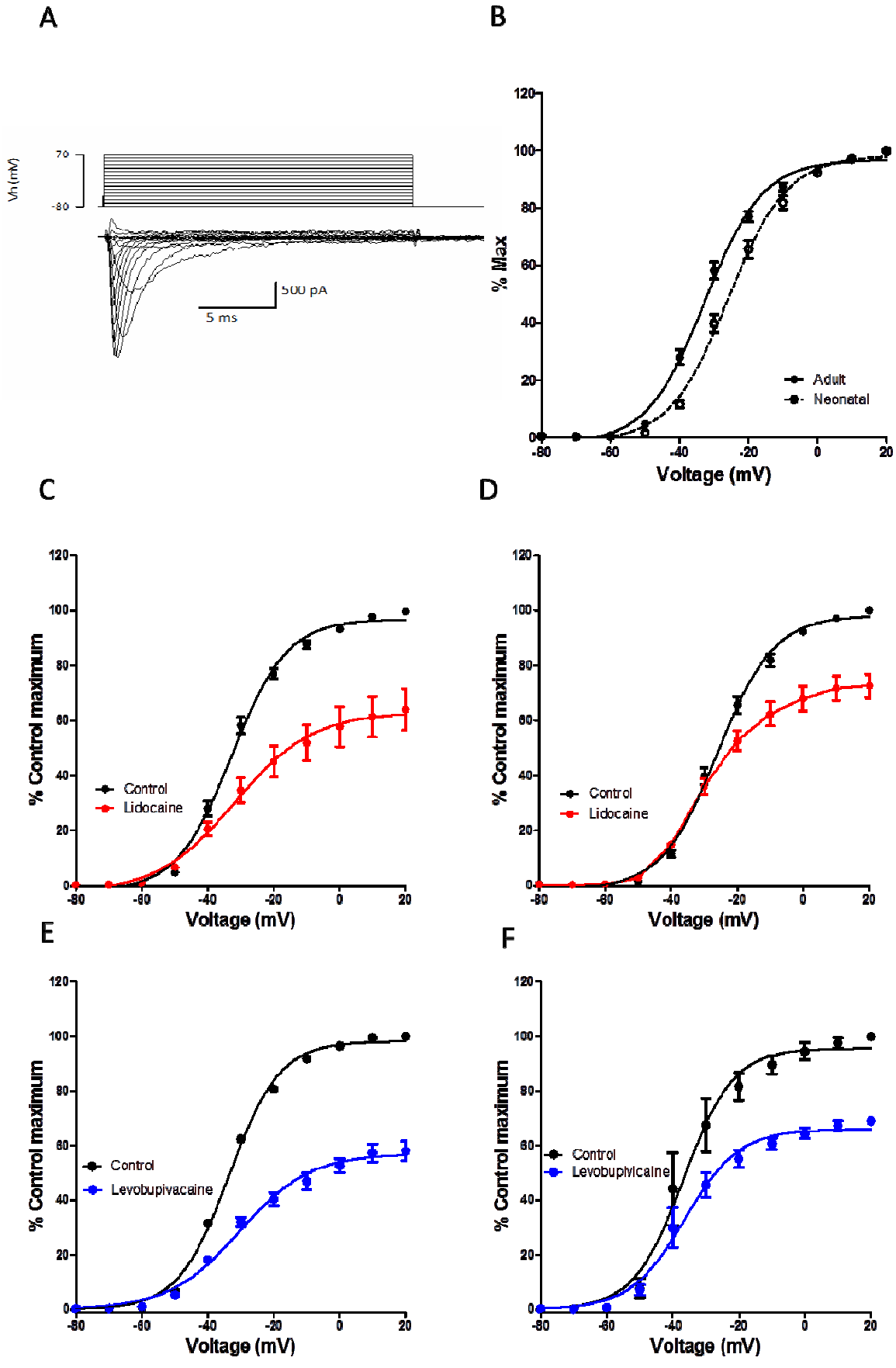
A
Vh (mV)
70
-80
500 pA
5 ms
B
% Max
Adult
Neonatal
Voltage (mV)
C
% Control maximum
Control
Lidocaine
Voltage (mV)
D
% Control maximum
Control
Lidocaine
Voltage (mV)
E
% Control maximum
Control
Levobupivacaine
Voltage (mV)
F
% Control maximum
Control
Levobupivicaine
Voltage (mV)

Figure 3.2 The effects of lidocaine and levobupivacaine on the voltage-dependence of activation of adult and neonatal $Na_V1.5$. Conductances obtained from the current-voltage protocol were normalised to their respective peak values, and plotted against voltage to establish the activation curve. Data points were normalised to peak amplitudes under control conditions. (A) The voltage-protocol with representative currents recorded from HEK293 expressing adult $Na_V1.5$ channels, from which the voltage-dependence of activation was established. Curves were fitted with Boltzmann functions and average parameters are summarised in Table 4 (B) The voltage-dependence of activation of currents mediated by adult (solid line) and neonatal (dotted line) $Na_V1.5$ channels recorded at -80 mV. (C) and (D) represent the voltage-dependence of activation in the absence and presence of lidocaine (10 μM), of adult and neonatal $Na_V1.5$, respectively. (E) and (F) represent the voltage-dependence of activation in the absence and presence of levobupivacaine (1 μM), of adult and neonatal $Na_V1.5$, respectively. Both lidocaine and levobupivacaine suppress the conduction of both splice variants over potentials between -80 mV and +20 mV, but cause no changes to the voltage-dependence of activation.

3.3 The effects of lidocaine and levobupivacaine on the voltage-dependence of inactivation of adult and neonatal $Na_V1.5$

Iexamined the influence of lidocaine and levobupivacaine on the voltage-dependence of inactivation of currents mediated by $Na_V1.5$. We applied 100 ms prepulses from -140 mV to -10 mV, followed by an activating step to 0 mV and examined the proportion of channels that are available for activation. The derived inactivation curves are shown in figure 3.3.B. As previously reported, there were no significant differences in the voltage-dependence of inactivation between the adult and the neonatal splice variants (Table 3.2). Both local anaesthetics caused a significant shift in the $V_{1/2}$ of inactivation of currents mediated by both adult and neonatal NaV1.5 VGSCs to more hyperpolarised potentials. No differences were observed between splice variants with respect to the shift in the V1/2 of inactivation. Interestingly, compared to levobupivacaine, lidocaine caused less inhibition at the more hyperpolarised potentials from -140 mV to -100 mV (Figure 3.3.C,E). I also examined the influence of lidocaine and levobupivacaine on the voltage-dependence of inactivation at a holding potential of -120 mV, where inactivation is absent. No inhibition or shift in the $V_{1/2}$ of inactivation were observed in the presence of either LA (Figure 3.4).

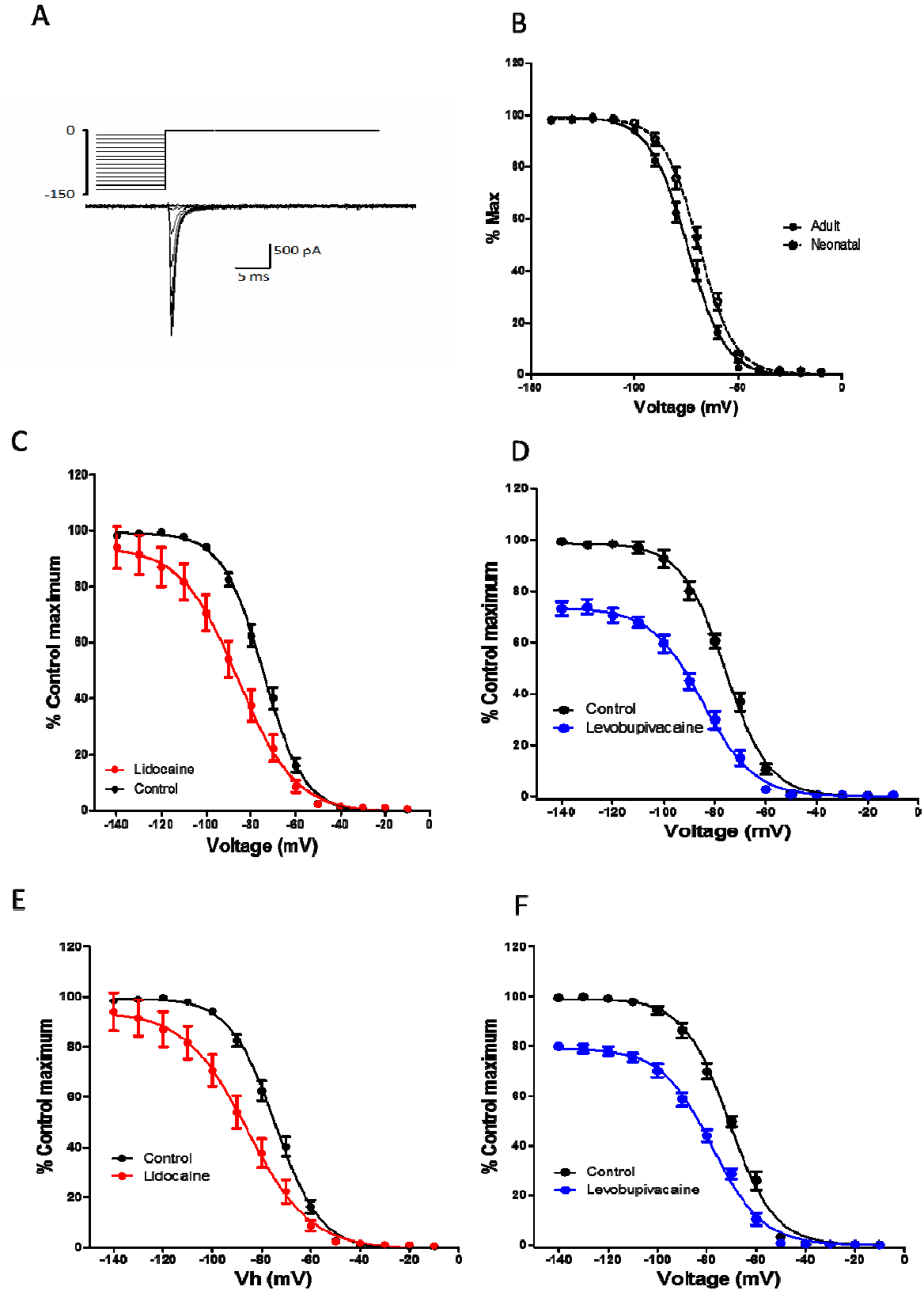
A
0
-150
500 pA
5 ms
B
% Max
Adult
Neonatal
Voltage (mV)
C
% Control maximum
Lidocaine
Control
Voltage (mV)
D
% Control maximum
Control
Levobupivacaine
Voltage (mV)
E
% Control maximum
Control
Lidocaine
Vh (mV)
F
% Control maximum
Control
Levobupivacaine
Voltage (mV)

Figure 3.3 Lidocaine and levobupivacaine influence the voltage-dependence of inactivation of adult and neonatal $Na_V1.5$. Current amplitudes recorded from the steady-state inactivation protocol were normalised to their respective peak amplitudes and plotted against voltage to establish the inactivation curve. Curves were fitted with Boltzmann functions and average parameters are summarised in Table 4 (A) The steady-state inactivation protocol with representative currents mediated by adult $Na_V1.5$ channels, from which the voltage-dependence of inactivation was established (B) The voltage-dependence of inactivation of currents mediated by the adult (solid line) and neonatal (dotted line) $Na_V1.5$ channels. (C) and (D) represent the voltage-dependence of inactivation in the presence and absence of lidocaine (10 μM), of adult and neonatal $Na_V1.5$, respectively. (E) and (F) represent the voltage-dependence of inactivation in the presence and absence of levobupivacaine (1 μM), of adult and neonatal $Na_V1.5$, respectively. Lidocaine and levobupivacaine significantly caused a hyperpolarising shift to the voltage-dependence of inactivation of adult and neonatal $Na_V1.5$ ($p < 0.05$, paired t-test).

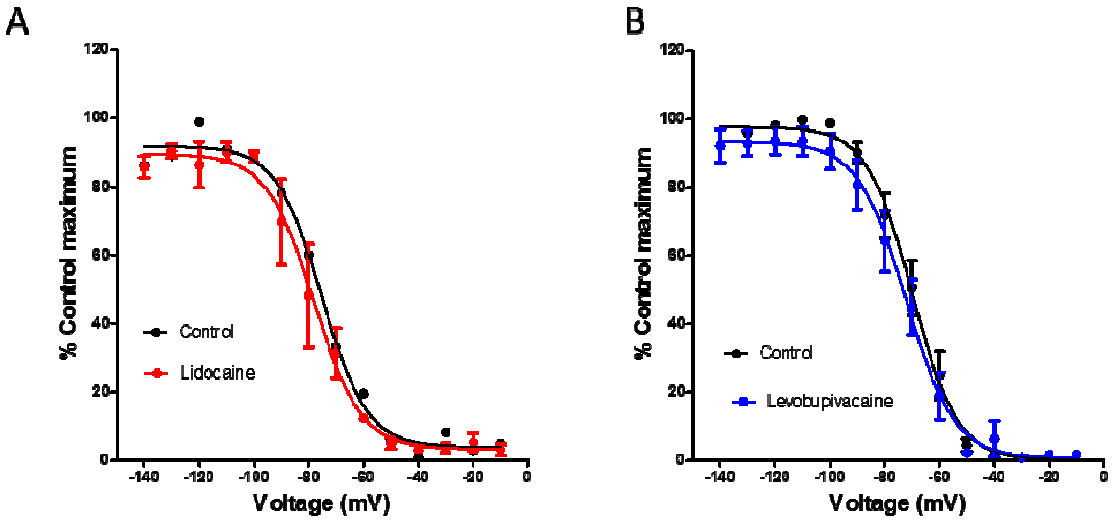

Figure 3.4 The effects of lidocaine and levobupivacaine on the voltage-dependence of inactivation at -120 mV. Voltage-dependence of inactivation and Boltzmann fits were performed as previously described in Figure 3.2. Lidocaine and levobupivacaine showed no inhibition at -120 mV and had no effect on the voltage-dependence of inactivation of currents mediated by the adult $Na_V1.5$.

Parameter		**Activation $V_{1/2}$**	**Activation slope**	**Inactivation $V_{1/2}$**	**Inactivation slope**	**n**
Adult $Na_V1.5$	**Control**	-33 ± 2.0	8.0 ± 0.3	-74 ± 2.0	8.0 ± 0.4	14
	Lidocaine	-32 ± 8.0	11.1 ± 0.9*	-87 ± 3.0*	12.0 ± 0.7*	14
	Levobupivacaine	-30 ± 0.9	11.0 ± 0.4*	-85 ± 1.3*	11.0 ± 0.4	3
Neonatal $Na_V1.5$	**Control**	-26 ± 1.0	8.0 ± 0.2	-69 ± 2.0	8.0 ± 0.3	11
	Lidocaine	-29 ± 0.9	8.0 ± 0.5	-78 ± 2.0*	11.0 ± 0.5*	11
	Levobupivacaine	-37 ± 3.8	7.0 ± 0.8	-78 ± 1.0*	10.0 ± 0.7*	3

Table 3.2 Summary of electrophysiological parameters of adult and neonatal $Na_V1.5$ in the absence and presence of lidocaine and levobupivacaine. $V_{1/2}$ and slope values were taken from the Boltzmann fits to data from individual cells. Statistical analyses were performed using a paired t-test. Asterisks represent statistically significant differences, ≤ p 0.05.

3.4 Resting-state and use-dependent block by lidocaine and levobupivacaine

Local anaesthetics are sensitive to the conformational state of VGSCs. Their blockade of VGSCs in nociceptive neurons is use-dependent. We investigated state-dependence for lidocaine and levobupivacaine at three holding potentials: -80 mV, at which a large proportion (approximately 40 %) of the channels are in the inactivated state (Figure 3.3.B), -90 mV, which is the approximate cardiac resting membrane potential; and -120 mV, where inactivation is absent. Initially, I determined the time period for lidocaine and levobupivacaine to reach steady-state inhibition using a single sweep protocol where LAs were applied during stimulation. For both LAs, a 2-minute incubation was sufficient to reach steady-state inhibition. In order to examine resting-state block currents were elicited by an activating pulse to 0 mV at 1 Hz before and after a 2-minute drug perfusion under no stimulation. The extent of inhibition on the first sweep after steady-state revealed that resting-state inhibition was influenced by the holding potential. Both lidocaine and levobupivacaine caused the largest inhibition at -80 mV, where inactivation is approximately half-maximal. Inhibition at -90 mV, where a small proportion of channels are inactivated, was minimal. By contrast, no inhibition occurred in the absence of inactivation at -120 mV. Furthermore, no differences were observed between the two splice variants with respect to resting-state inhibition (Figure 3.5.B,C).

Use-dependent inhibition was determined by examining the accumulation of block in the subsequent sweeps after stimulation was resumed (at 1 Hz).

Lidocaine showed no use-dependence at -80 mV, presumably because steady-state inhibition had already reached equilibrium in the absence of stimulation. Likewise, no systematic use-dependence was observed at -120 mV where inhibition was negligible, perhaps due to no accumulation of inactivation at 1 Hz. By contrast, lidocaine showed inhibition in a use-dependent manner at -90 mV, only on the adult splice variant (Figure 3.6.A). In comparison, levobupivacaine inhibited both splice variants at -80 mV and -90 mV in a use-dependent manner (Figure 3.6.C, D) No systematic use-dependence was observed for levobupivacaine at -120 mV.

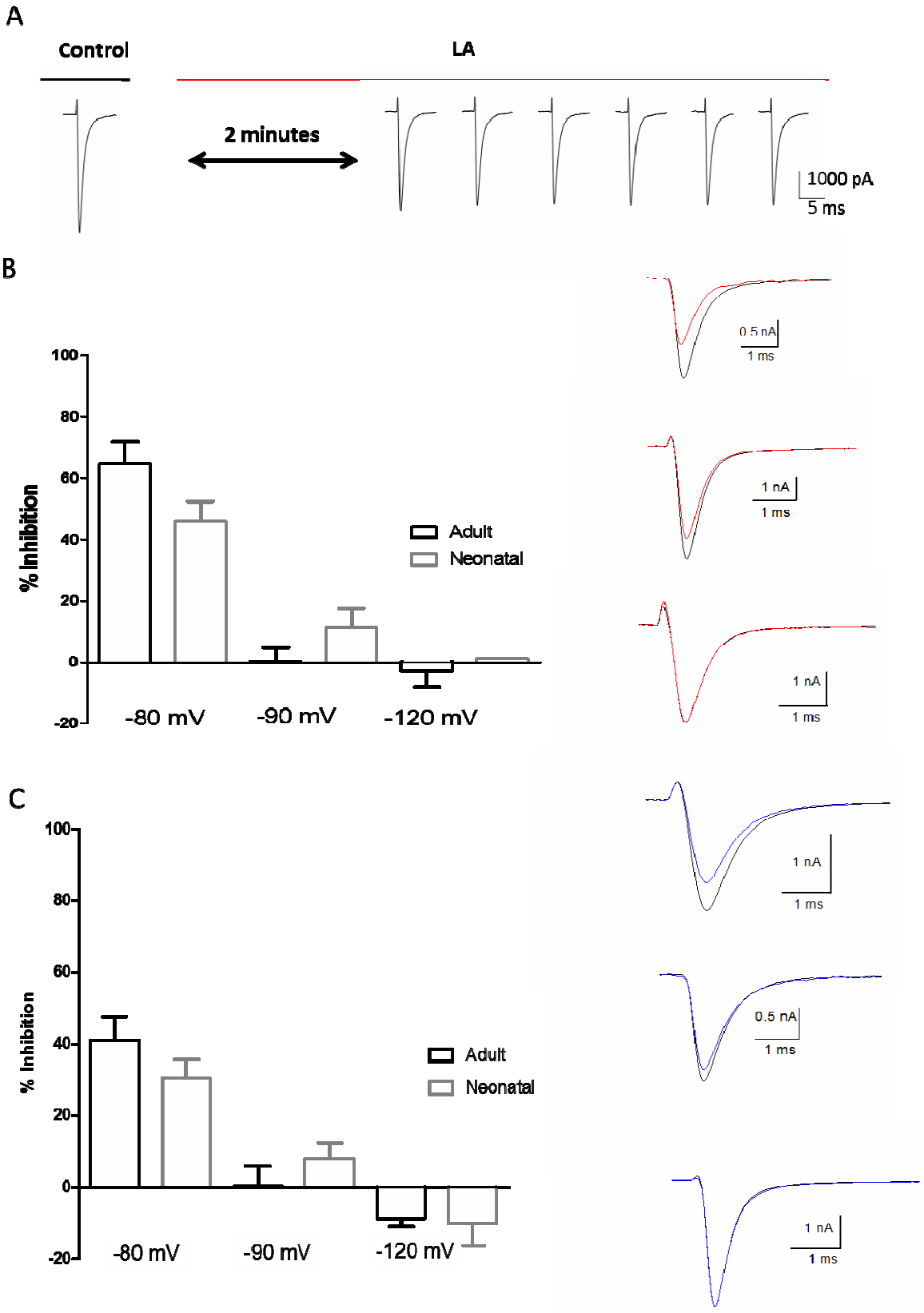
A
Control
LA
2 minutes
1000 pA
5 ms
B
% Inhibition
100
80
60
40
20
0
-20
Adult
Neonatal
-80 mV
-90 mV
-120 mV
0.5 nA
1 ms
1 nA
1 ms
1 nA
1 ms
C
% Inhibition
100
80
60
40
20
0
-20
Adult
Neonatal
-80 mV
-90 mV
-120 mV
1 nA
1 ms
0.5 nA
1 ms
1 nA
1 ms

Figure 3.5 Lidocaine and levobupivacaine inhibit adult and neonatal $Na_V1.5$ in a state-dependent manner. (A) Graphical illustration of the protocol used to examine resting-state and use-dependent inhibition with exemplary currents mediated by adult $Na_V1.5$ channels at -90 mV. Inhibition by lidocaine (B) and levobupivacaine (C) was determined at three different holding potentials: -80, -90 and -120 mV. Inhibition was determined at rest, i.e. in the absence of stimulation by applying the drugs for 2 minutes to reach steady-state inhibition, then activating the channels to 0 mV. Current amplitudes were then expressed as a percentage of the average of 5 sweeps recorded prior to drug application. For both drugs, the largest inhibition was observed at -80 mV, for which potential there is significant inactivation. By contrast to -80 mV, inhibition -90 mV was minimal. There was no inhibition at -120 mV, a potential at which there is no inactivation. No significant differences were observed between the splice variants with respect to resting-state inhibition at any of the potentials examined. Inset represents exemplary currents mediated by adult $Na_v1.5$ at the tested holding potentials. Red and blue traces were recorded in the presence of lidocaine and levobupivacaine, respectively.

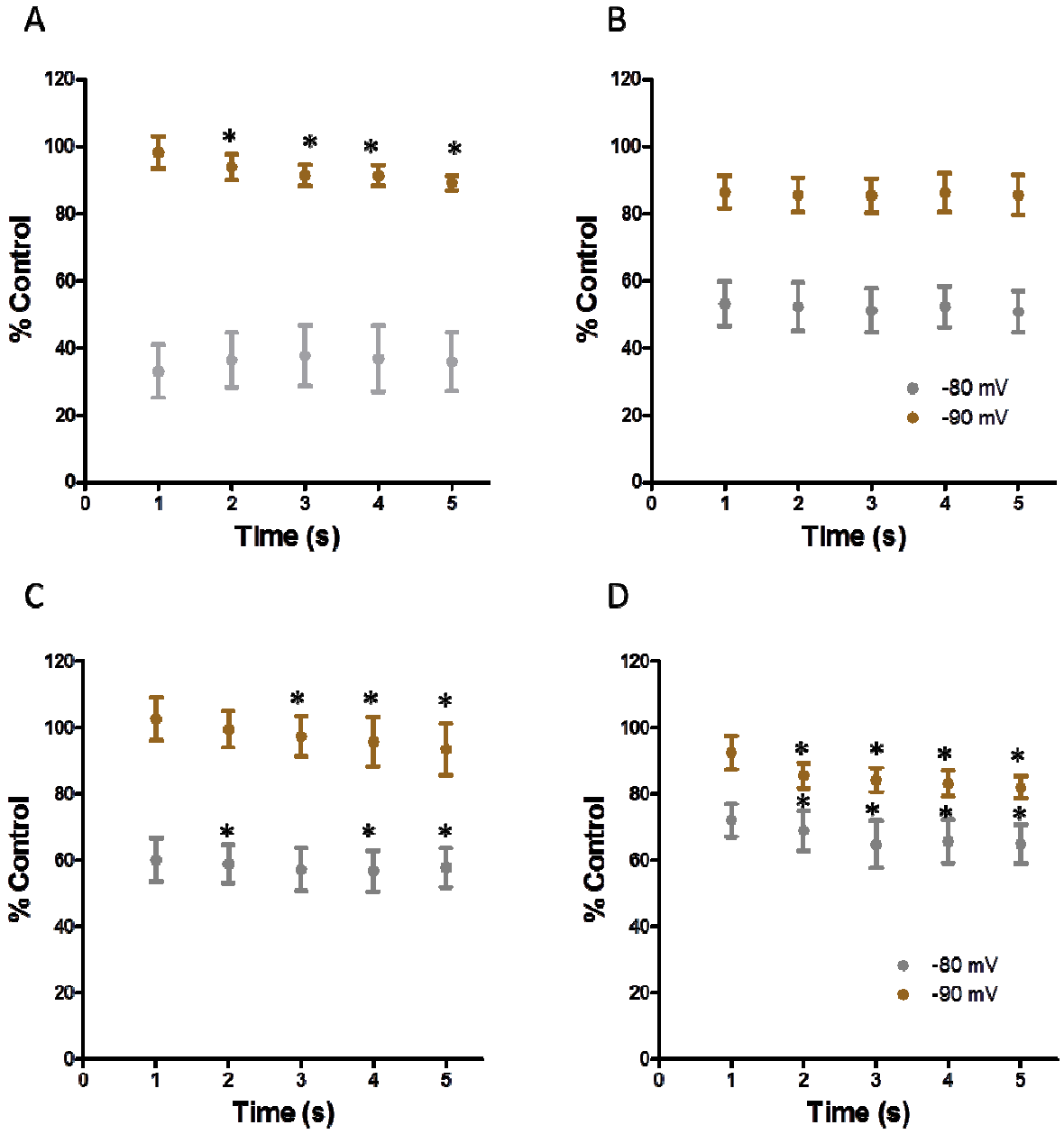
A
B
C
D
% Control
Time (s)
-80 mV
-90 mV

Figure 3.6 Use-dependent block by lidocaine and levobupivacaine of adult and neonatal $Na_V1.5$ at -80, -90 and -120 mV.Control currents were recorded prior to a 2 minute perfusion with local anaesthetics in the absence of stimulation. Stimulation was then resumed at 1 Hz for 10 seconds. The graphs represent mean current amplitudes recorded for 5 seconds after stimulation was resumed, normalised to their respective control values, to take systematic run down into consideration. (A) and (B) Use-dependent inhibition by lidocaine of currents mediated by adult and neonatal $Na_V1.5$, respectively. (C) and (D) Use-dependent inhibition by levobupivacaine of currents mediated by adult and neonatal $Na_V1.5$, respectively. Lidocaine displayed use-dependent blockade of adult $Na_V1.5$ at a holding potential of -90 mV. Levobupivacaine displayed use-dependent inhibition of both adult and neonatal $Na_V1.5$ at holding potentials of -80 and -90 mV. Mean % control values were compared to the first data point after drug perfusion using a repeated measures ANOVA, followed by a Dunnett's multiple comparisons test. Asterisks represent statistically significant differences, ≤ p 0.05.

3.5Recovery from inactivation and block by lidocaine and levobupivacaine

The differential effects of lidocaine and levobupivacaine on adult and neonatal $Na_V1.5$ may arise from their different unbinding kinetics. Therefore, we used a protocol designed to examine the rate of recovery from inactivation of the two splice variants, in the presence and absence of lidocaine and levobupivacaine. We used a holding potential of -80 mV to induce inactivation and hence enable inhibition. After that a step to -120 mV was applied for durations between 10 to 300 ms to relieve inactivation and allow recovery from inactivation. In the absence of LAs, the rate of recovery from inactivation at -80 mV appeared slower for the neonatal splice variant compared to the adult (Figure 3.7.B), although no significant differences were observed between the time constants (Table 3.3).

The extent of recovery from inactivation in the presence of lidocaine compared to levobupivacaine was markedly different (Figure 3.8). Currents mediated by the adult splice variant treated with lidocaine recovered by 82 ± 4 % (n=6), whereas those treated with levobupivacaine recovered by 61 ± 2 % (n=6) (Figure 3.8.A, B). Lidocaine influenced the recovery rate of the adult splice variant, which revealed a fast component that was slowed by lidocaine, and a slow one that was not affected. The time constants of recovery were 4.0 ± 0.9 ms (n=3) and 6.0 ± 0.7 ms (n=6), in the absence and presence of lidocaine, respectively. Conversely, lidocaine did not affect the rate of recovery from block of the neonatal splice variant, which only revealed a single fast component of 4 ± 0.6 ms (n=4) and 5 ± 0.6 ms (n=6) in the presence and absence of lidocaine, respectively. Recovery from block by levobupivacaine was similar for both splice variants with weighted time constants of 17 ± 8 ms (n=3) and 13 ± 4 ms (n=3), for adult and neonatal, respectively. The time constants of recovery are summarised in Table 5.

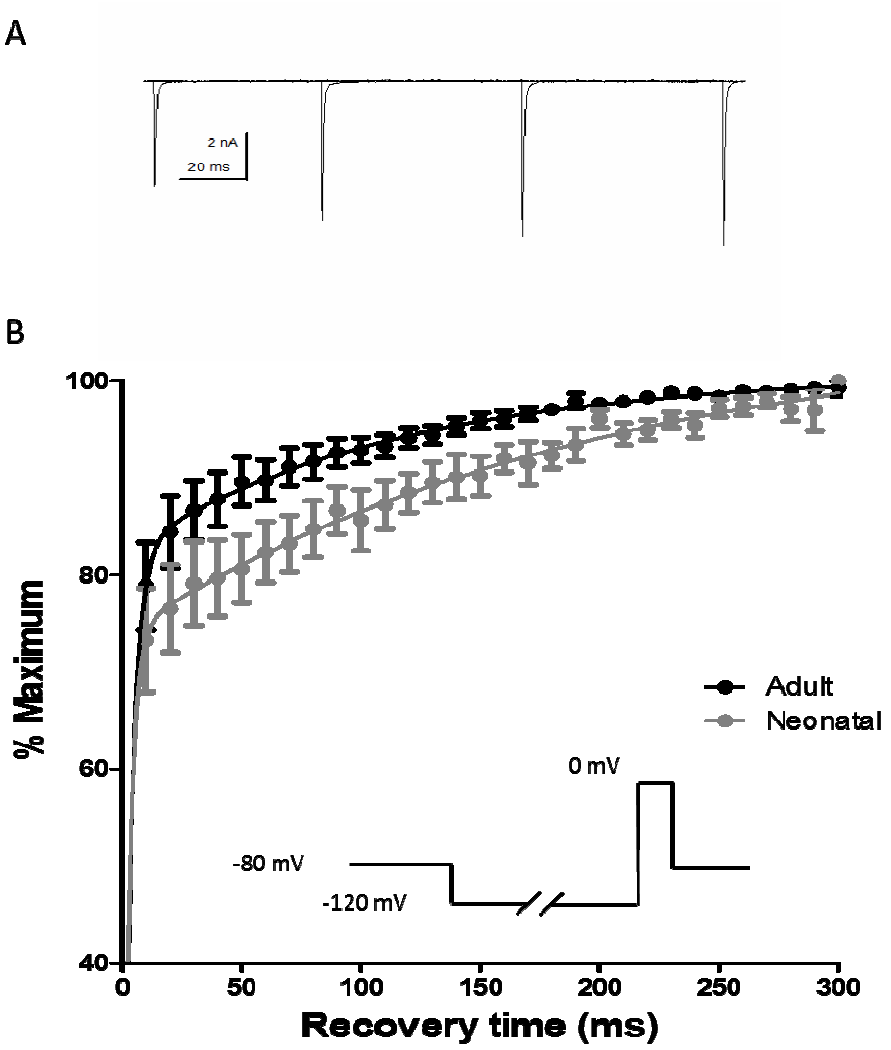

Figure 3.7 Recovery from inactivation at -80 mV of adult and neonatal $Na_V1.5$. (A) The rate of recovery from inactivation at -80 mV of adult and neonatal $Na_V1.5$ induced by a hyperpolarising step to -120 mV for durations between 10 and 300 ms. Currents were normalised to peak values from individual cells. Data points were fitted with a double exponential decay function. Average parameters obtained from data points taken from individual cells are summarised in Table 3. Inset represents exemplary currents mediated by adult $Na_V1.5$ in HEK293 cells.

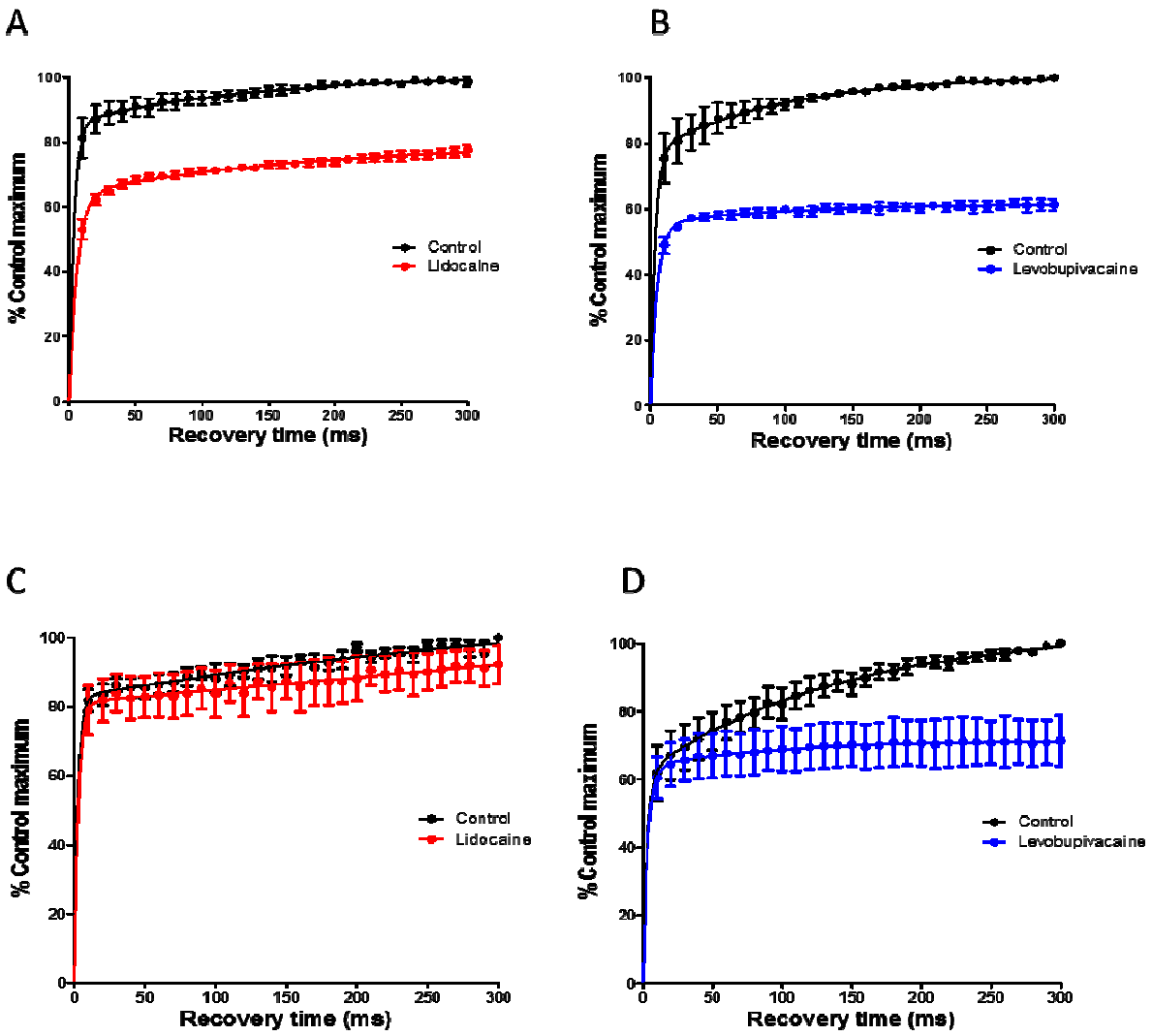

Figure 3.8 Recovery from inactivation of adult and neonatal $Na_V1.5$ in the presence of lidocaine and levobupivacaine. (A) and (B) represent the rate of reversal of lidocaine block of adult and neonatal $Na_V1.5$, respectively. (B) and (C) represent the rate of recovery from levobupivacaine block of adult and neonatal $Na_V1.5$, respectively. Paired recordings were made before and during steady-state inhibition. Reversal of inhibition was induced by a hyperpolarising step to -120 mV for durations between 10 and 300 ms. Currents were normalised to peak values under control conditions.

Parameter		Slow time constant (ms)	Fast time constant (ms)	Fast component amplitude (%)	Weighted time constant (ms)	n
Adult $Na_V1.5$	**Control**	159 ± 28	4 ± 0.9	81 ± 5	30 ± 8	6
	Lidocaine	152 ± 54	6 ± 0.7*	81 ± 3	39 ± 15	3
	Levobupivacaine	99 ± 37	5 ± 0.9	88 ± 3	17 ± 8	3
Neonatal $Na_V1.5$	**Control**	168 ± 19	4 ± 0.6	77 ± 8	41 ± 12	6
	Lidocaine	N/A	5 ± 0.6	100	4 ± 0.5*	4
	Levobupivacaine	87 ± 11	4 ± 0.2	88 ± 5	13 ± 4	3

Table 3.3. Summary of the time constants of recovery from inactivation and block by lidocaine and levobupivacaine. Time constants were taken from either single or double exponential fits to data from individual cells. Statistical analyses were performed using an unpaired t-test. Asterisks represent statistically significant differences, ≤ p 0.05. Comparisons were made between the time constants in the presence of LAs and the controls.

4. Discussion

4.1 Summary

Lidocaine and levobupivacaine both potently inhibit currents mediated by adult and neonatal $Na_V1.5$, with levobupivacaine being approximately 10-fold more potent than lidocaine (Table 3). Neither LA showed selective inhibition for either splice variant, or differences with respect to the concentration-dependence of inhibition. They differ in their mode of action on adult and neonatal $Na_V1.5$ as follows. Levobupivacaine is more potent than lidocaine and it inhibits both splice variants in a use-dependent manner at a stimulus frequency of 1 Hz, unlike lidocaine, which only exhibits use-dependent inhibition on the adult splice variant at -90 mV. Moreover, inhibition by levobupivacaine is irreversible by hyperpolarisation. Conversely, inhibition by lidocaine is readily reversible and splice variant dependent, whereby the rate of recovery of the neonatal splice variant is faster than that of the adult. These preclinical findings provide useful information for designing a suitable regimen for using LAs as anti-metastatic drugs. In addition, understanding the mechanism of action of LAs on the neonatal splice variant has important applications in the practice of anaesthesia for neonates.

4.2 Technical considerations

In this study, the mechanism of action of lidocaine and levobupivacaine was investigated on adult and neonatal $Na_V1.5$, which are both expressed in metastatic colon cancer cells. Although whole-cell recordings can be made from the model metastatic cancer cells (SW620), the recombinant system was chosen for this study as a means to distinguish between the two splice variants, since no selective antagonists are available for either splice variant.

However, the recombinant $Na_V1.5$ may not reflect the full electrophysiological properties of those in the cancer cells, due to the absence of modulatory subunits, such as beta- subunits and other accessory proteins that may influence the biophysical properties, as well as the pharmacology of these channels. In addition, HEK293 cells express endogenous Na_V channels when passaged multiple times in culture. Therefore, it is vital not to get contamination from these channels and therefore, HEK293 cells were only recorded from up to a passage number of 40.

The LAs used in this study are state-dependent blockers that require inactivation of the channels in order to cause their inhibitory effects. Since inactivation is determined by the voltage, I ensured that the p/6 routine used for leak subtraction was carried out after each sweep, to ensure that the drugs do not unbind as a result of hyperpolarisation and the subsequent removal of inactivation.

The concentration used in this study, lidocaine (10 μM) and levobupivacaine (1 μM) correspond to those achieved systemically during regional anaesthesia. Lidocaine has been shown to inhibit invasion in vitro. However, to date there is no evidence the inhibition of invasion by levobupivacaine, which has proven problematic when used in the matrigel invasion assay (unpublished data). This study would be strengthened by including data from the matrigel invasion assay with levobupivacaine.

4.3The mode of action of lidocaine and levobupivacaine on adult and neonatal $Na_V1.5$

Consistent with previous findings, both LAs exhibited state-dependent inhibition[73,81]. This is evident from the relationship between the holding potential, which dictates channel conformation, and the amount of inhibition (Figure 3.5.B,C). The extent of inhibition is correlated with the proportion of channels in the inactivated state. At -80 mV, where approximately 40% of channels are inactivated, the inhibition is larger than that at -90, where only

20% of the channels are inactivated (Figure3.3.B). Further to that, both LAs caused a significant shift in the voltage-dependence of inactivation, whereas neither LA had an effect on the voltage-dependence of activation (Table 4). This suggests that the inactivated state is required for LA action, and is stabilised in the presence of lidocaine and levobupivacaine.

In the absence of inactivation (-120 mV), lidocaine (10 μM) and levobupivacaine (1μM) both failed to inhibit currents mediated by adult and neonatal $Na_V1.5$. Thus, it follows that removal of inactivation should relieve the inhibition caused by LAs. I examined the recovery from blockade by LAs by using a hyperpolarising step to -120 mV to remove inactivation. The results from these experiments revealed differential modes of recovery from inhibition by lidocaine and levobupivacaine. Within 300 ms the extent of recovery from inhibition by lidocaine is larger than that of levobupivcaine (Figure3.8). This suggests that lidocaine unbinds as the channels recover from inactivation, while levobupivacaine remains bound despite recovery from inactivation within the time scale tested. Interestingly, the unbinding mode of lidocaine appears to differ between the two splice variants. The extent of recovery of the neonatal splice variant from lidocaine blockade is greater than that of the adult splice variant. A striking observation was that the time constant of recovery from inactivation of the neonatal splice variant only revealed a single component. This may suggest that lidocaine does not affect slow inactivation of the neonatal splice variant. By contrast, levobupivacaine displays a uniform mode of unbinding from both splice variants. Levobupivacaine's inability to unbind may be due to the different route by which it gains access to its binding site, resulting in trapping and failure to unbind upon recovery from inactivation. This observation is also reflected in the steady-state inactivation data. At hyperpolarised potentials, where inactivation is absent, lidocaine seems to cause less inhibition compared to levobupivacaine. This is due to unbinding of lidocaine within the 100 ms pre-pulse and failure of levoubuipvacaine to unbind within the same duration.

Lidocaine and levobupivacaine differ also in their use-dependent inhibition. While lidocaine only showed use-dependent inhibition at a holding potential

of -90 mV on the adult splice variant, levobupivacaine inhibited both splice variants in a use-dependent manner at holding potentials of -80 and -90 mV. This is perhaps also a consequence of failure to unbind, which leads to accumulation of levobupivacaine upon depolarisation leading to increased inhibition. However, in the case of lidocaine, no clear explanation can be offered thus far, as the unbinding kinetics at -90 mV may differ between the two splice variants, which may underlie the differential use-dependence. At -80 mV, inhibition seems to have reached steady-state during the 2 minute perfusion period and thus no further inhibition occurs upon depolarisation.

4.4Hydrophobicity and LAs mechanism of action

Amide-linked LAs share a common structure of an aromatic ring, a tertiary amine group and an intermediate amide linkage. Lidocaine differs from levobupivacaine in the tertiary amine group, where it is part of diethyleamine in the former and a butylpiperidinein the latter[82]. Such chemical differences reflect on the pharmacokinetic and pharmacodynamics profile of LAs. This study suggests that lidocaine unbinds more readily from inactivated $Na_V1.5$ channels compared to its counterpart, levobupivacaine. The large butylpiperidinegroup renders levoubupivacaine more hydrophobic than lidocaine. Hydrophobicity largely influences the potency of LAs at the tissue and the molecular level. The following postulations may explain the differential unbinding profile of lidocaine and levobupivacaine. Firstly, it is known that hydrophobic LAs can readily gain access into the binding site via the fenestrations within the lipid bilayer[69]. Secondly, hydrophobic molecules tend to get trapped within hydrophobic pockets[83]. Such properties of LAs are vital to take into consideration in rational drug design.

4.5Scope for selectivity

Although VGSCs are ubiquitously expressed in excitable tissues, they exist as distinct populations of channels with unique sensitivities to LAs. Inactivated VGSCs, which are highly sensitive to LAs have a short half-life

under physiological conditions, as the channels transition between closed, open and inactivated conformations. However, under pathological conditions, e.g. pain and in the case of metastatic colon cancer cells, which have a relatively more depolarised membrane potential[29,84], the inactivated state has a longer half-life and thus the channels are more susceptible to blockade by LAs. Furthermore, the inhibitory effects of LAs may reduce I_{NaP}, which is thought to contribute to cancer cell invasiveness[27]. Such properties of state-dependent blockers confer a therapeutic advantage for LAs and allow for discrimination between the same molecular targets under different conditions. In addition to the potential benefits of local anaesthesia discussed earlier[8-10], LAs at concentrations that are sufficient to block $Na_V1.5$ activity[29] correspond to those given at clinical doses[82], which makes them ideal candidates for antimetatatic drugs.

Given the subtle differences between adult and neonatal $Na_V1.5$ with respect to kinetics and voltage-dependence of gating[29,30], along with the identical drug binding sites; it is challenging to design selective molecules for the neonatal splice variant. This puts a limit on the range of selectivity of pharmacotherapy leading researchers to explore alternative avenues. Antibody therapy may offer possibilities of selectivity by using antibodies against the neonatal splice variant. Chioni et al have developed a polyclonal antibody that selectively binds the neonatal splice variant, named NESOpAb[85], which has also been shown to block neonatal $Na_V1.5$ activity when applied extracellularly at concentrations of 0.05 ng/ml. Those findings offer promising therapeutic applications of NESOpAb for neonatal $Na_V1.5$ pathological expression in adult metastatic cancers.

4.6 Clinical implications:

Given the importance of VGSCs in cancer cell invasiveness, numerous in vitro and vivo studies have shown that inhibition of VGSC function can mitigate metastasis and thus such drugs can be used clinically as antimetastatic agents. LAs are one example, which have also been shown in retrospective studies to influence the outcome of surgery and reduce the incidence of recurrence (see introduction). To that end, LAs, such as

lidocaine and levobupivacaine can be re-purposed as antimetastatic drugs. Their use is particularly important during tumour resection where they can either be injected directly onto the tumour, or perfused intravenously. For the latter route of administration, systemic toxicity is one important consideration, especially cardiotoxicity as the adult $Na_V1.5$ is functionally expressed in cardiomyocytes. According to this study, it can be inferred that lidocaine would be more suitable to be given intravenously, as it readily unbinds and hence would be more cardiac sparing. Whereas levobupivacaine, which is more potent than lidocaine, may be used for direct injection onto the tumour, where the microenvironment is more acidic due to inflammation. That would ensure that sufficient amounts are achieved locally to inhibit the VGSCs in the cancer cells with minimal leak to the circulation, since levobupivacaine is likely to be more cardiotoxic given that it fails to unbind from the channels.

Failure of LA molecules to unbind may lead to accumulation within the channel. Thus, it can be inferred that irreversible blockade could potentially lead to toxicity, especially in cells that repetitively fire action potentials, e.g. cardiomyocytes. Bupivacaine, which is a racemic mixture, is reported to accumulate in guinea pig cardiomyocytes and thus cause cardiotoxicity[86]. Although levobupivacaine is reported to be less toxic than bupivacaine, it is likely to cause cardiotoxicity at physiological heart rates (60-120 beats per minute), where the diastolic period is not sufficient for complete recovery from blockade. Lidocaine, on the other hand would be more cardiac sparing as it unbinds relatively quickly, and is hence safer to use intravenously either peri- or post-operatively in tumour resection.

These have important implications in the clinical use of these LAs in cancer patients who may suffer from heart conditions, such as angina, where pathological changes may influence the resting membrane potential and hence affect the degree of inhibition and the unbinding mode of these LAs. Moreover, the findings from this study are also relevant for the use of lidocaine in neonates, since it exhibits a differential mode of unbinding and therefore considerations must be taken with respect to dosing regimens.

In addition to the use of LAs during tumour resection, they can be given as adjuvant drugs along with the conventional therapies for cancer for better management. Their clinical potential to ameliorate cancer metastasis may aid in turning cancer into a chronic condition. Since these drugs are already used clinically, there would be no issues with dosage, side effects and long-term use. Nevertheless, new compositions of LAs and other VGSC inhibitors, particularly I-NaP inhibitors, e.g. ranolazine and riluzole can be modified to reduce their off target effects and tailor them to target VGSCs in cancer cells.

5. Future work

5.1Local anaesthesia in tumour resection

LAs can either be administered directly into the tumour, or infused intravenously peri-, or post-operatively in an attempt to mitigate metastasis. Levobupivacaine coapplied with adrenaline would be suitable for the former method, but not the latter, for which lidocaine would be preferred due to the previously discussed properties of inhibition. Interestingly, the activity of the neonatal $Na_V1.5$ in metastatic breast cancer cells MDA-MB-231increases mRNA levels and membrane expression of the channels via a PKA-mediated positive feedback loop[58]. Therefore, it is conceivable that inhibition of VGSC would have a dual advantage of inhibiting their activity and surface expression.

Although it is established that LAs inhibit cell invasion *in vitro*[29,41]and that other VGSC blockers reduce metastasis *in vivo*[61,68], it remains unclear whether the therapeutic benefits of LAs are directly due to inhibition of VGSCs. To date, LAs have not been tested on animal models of metastasis, of which there are few. There are no available animal models for metastatic colon cancer; however, the Dunning rat model for prostate cancer can be used as a model for metastasis[87].

5.2The effects of lidocaine and levobupivacaine on fast and slow inactivation

It is unclear from this study whether fast and slow inactivation and their modulation by LAs are different between the two splice variants. The steady-state inactivation protocol used in this study examines fast inactivation. Slow inactivation, however, occurs in response to prolonged depolarisations. Therefore, to address this question the following protocol must be carried out. In order to examine slow inactivation a long conditioning pulse must be applied for 5 seconds before a hyperpolarising step to -120 mV to remove fast inactivation. Currents can then be elicited by a step to 0 mV to examine the available current in the presence and absence of LAs. Furthermore, the recovery protocol used in this study examines recovery from closed-state inactivation at -80 mV. In order to examine recovery from fast inactivation, a paired pulse protocol can be used, where a conditioning pulse is applied initially to drive the channels into fast inactivation, after which a step to -120 mV is applied for varying durations. A second test pulse to 0 mV can then be applied to measure the recovered current. The same protocol can also be used to examine recovery from inactivation at -90 mV, as the differential use-dependent inhibition by lidocaine on adult and neonatal $Na_V1.5$ may arise from different recovery kinetics at -90 mV.

5.3Time course of recovery from local anaesthetic blockade

Once high affinity binding of LAs to inactivated channels occurs, unbinding can only take place upon recovery from inactivation, which frequently occurs in cardiomyocytes during diastole (relaxation of heart muscles). By contrast, in metastatic colon cancer cells, which have a membrane potential of approximately -40 mV, recovery from inactivation is infrequent and a high proportion of the channels remain inactivated. This consequently affects the reversibility of lidocaine block, suggesting that the function of $Na_V1.5$ channels may only be reinstated upon insertion of newly translated channels into the membrane. This could be tested by applying lidocaine to HEK293cellsrecombinanatly expressing $Na_V1.5$ channels and washing it off. The current density can then be measured over increasing duration of time.

A saturating concentration of TTX and a protein synthesis inhibitor can be used as positive and negative controls, respectively. These experiments would aid in establishing an appropriate dosing regimen.

5.4The Invasion machinery of metastatic colon cancer cells

The machinery of invasion in metastatic colon cancer cells remains elusive. The $Na_V1.5$ channels are suggested to act in concert with other cellular components to facilitate cell invasion. To this end, the Matrigel invasion assay can be used to elucidate the machinery of invasion of SW620 cells as models of metastatic colon cancer. Extracellular acidification via the interplay between sodium ions and proton exchange is one proposed mechanism that is well established in breast cancer cells (MDA-MB-231)[88]. $Na_V1.5$ and NHE1 colocalise at focal sites of membrane remodelling in invadopodia of MDA-MB-231 cells. Furthermore, NHE1 is allosterically regulated by $Na_V1.5$ activity; however, the mechanism remains unclear. The interplay between NaV1.5 and NHE1 leads to protonation of the extracellular environment, which favours the activity of cathepsins S and B, which subsequently break down the basement membrane. It is unclear whether this process is recapitulated in metastatic colon cancer cells. Another important ion exchange mechanism is calcium ion entry via the action of the sodium-calcium exchanger (NCX). Transcripts of the cardiac isoform NCX1 are detectable in SW620 cells (unpublished data). The activity of NHE1 and NCX1 can be inhibited using amiloride and nickel, respectively, and their involvement in SW620 cell invasion can be investigated using the Matrigel invasion assay. In addition to ion exchange mechanismsthat require sodium ion influx, the expression of $Na_V1.5$ alone may facilitate the transition to the metastatic phenotype through protein-protein interactions. One possible candidate is scotin[89], which is a p53-inducable pro-apoptotic protein that is normally expressed in the nuclease and the endoplasmic reticulum. There is evidence that $Na_V1.5$ channels physically interact with scotin (Baptista-Hon and Hales, unpublished data), and thus colocalise in the cell surface where scotin can no longer execute its pro-apoptotic function. These primary observations need validation and further investigation.

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